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(ST) Abstract

The present invention relates generally to the control of body weight of animals including mammals and humans, and more particularly to materials identified herein as modulators of weight, and to the diagnostic and therapeutic uses to which such modulators may be put. In its broadest aspect, the present invention relates to the elucidation and discovery of nucleotide sequences, and proteins putatively expressed by such nucleotides or degenerate variations thereof, that demonstrate the ability to participate in the control of mammalian body weight. The nucleotide sequences in object represent





the genes corresponding to the murine and human OB gene, that have been postulated to play a critical role in the regulation of body weight and adiposity. Preliminary data, presented herein, suggests that the polypeptide product of the gene in question functions as a hormone. The present invention further provides nucleic acid molecules for use as molecular probes, or as primers for polymerase chain reaction (PCR) amplification, i.e., synthetic or natural oligonucleotides. In further aspects, the present invention provides a cloning vector, which comprises the nucleic acids of the invention; and a bacterial, insect, or a mammalian expression vector, which comprises the nucleic acid molecules of the invention, operatively associated with an expression control sequence. Accordingly, the invention further relates to a bacterial or a mammalian cell transfected or transformed with an appropriate expression vector, and correspondingly, to the use of the above-mentioned constructs in the preparation of the modulators of the invention. Also provided are antibodies to the OB polypeptide. Moreover, a method for modulating body weight of a mammal is provided. In specific examples, genes encoding two isoforms of both the murine and human OB polypeptides are provided.

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MODULATORS OF BODY WEIGHT, CORRESPONDING NUCLEIC ACIDS AND PROTEINS, AND DIAGNOSTIC AND THERAPEUTIC USES THEREOF

TECHNICAL FIELD OF THE INVENTION

5 The present invention relates generally to the control of body weight of mammals including animals and humans, and more particularly to materials identified herein as modulators of weight, and to the diagnostic and therapeutic uses to which such modulators may be put.

BACKGROUND OF THE INVENTION

- Obesity, defined as an excess of body fat relative to lean body mass, is associated with important psychological and medical morbidities, the latter including hypertension, elevated blood lipids, and Type II or non-insulin-dependent diabetes melitis (NIDDM). There are 6-10 million individuals with NIDDM in the U.S., including 18% of the population of 65 years of age [Harris et al., Int. J. Obes., 11:275-283 (1987)]. Approximately 45% of males and 70% of females with NIDDM are obese, and their diabetes is substantially improved or eliminated by weight reduction [Harris, Diabetes Care, 14(3):639-648 (1991)]. As described below, both obesity and NIDDM are strongly heritable, though the predisposing genes have not been identified. The molecular genetic basis of these metabolically related disorders
 is an important, poorly understood problem.
- The assimilation, storage, and utilization of nutrient energy constitute a complex homeostatic system central to survival of metazoa. Among land-dwelling mammals, storage in adipose tissue of large quantities of metabolic fuel as triglycerides is crucial for surviving periods of food deprivation. The need to maintain a fixed level of energy stores without continual alterations in the size and shape of the organism requires the achievement of a balance between energy intake and expenditure. However, the molecular mechanisms that regulate energy balance remain to be elucidated. The isolation of molecules that transduce nutritional information and

control energy balance will be critical to an understanding of the regulation of body weight in health and disease.

An individual's level of adiposity is, to a large extent, genetically determined. Examination of the concordance rates of body weight and adiposity amongst monoand dizygous twins or adoptees and their biological parents have suggested that the heritability of obesity (0.4-0.8) exceeds that of many other traits commonly thought to have a substantial genetic component, such as schizophrenia, alcoholism, and atherosclerosis [Stunkard et al., N. Engl. J. Med., 322:1483-1487 (1990)]. Familial similarities in rates of energy expenditure have also been reported [Bogardus et al., 10 Diabetes, 35:1-5 (1986)]. Genetic analysis in geographically delimited populations has suggested that a relatively small number of genes may account for the 30-50% of variance in body composition [Moll et al., Am. J. Hum. Genet., 49:1243-1255 (1991)]. However, none of the genes responsible for obesity in the general population have been genetically mapped to a definite chromosomal location.

15 Rodent models of obesity include seven apparently single-gene mutations. The most intensively studied mouse obesity mutations are the ob (obese) and db (diabetes) genes. When present on the same genetic strain background, ob and db result in indistinguishable metabolic and behavioral phenotypes, suggesting that these genes may function in the same physiologic pathway [Coleman et al., Diabetologia,
20 14:141-148 (1978)]. Mice homozygous for either mutation are hyperphagic and hypometabolic, leading to an obese phenotype that is notable at one month of age. The weight of these animals tends to stabilize at 60-70 g (compared with 30-35 g in control mice). ob and db animals manifest a myriad of other hormonal and metabolic changes that have made it difficult to identify the primary defect attributable to the
25 mutation [Bray et al., Am. J. Clin. Nutr., 50:891-902 (1989)].

Each of the rodent obesity models is accompanied by alterations in carbohydrate metabolism resembling those in Type II diabetes in man. In some cases, the severity of the diabetes depends in part on the background mouse strain [Leiter,

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Endocrinology, 124:912-922 (1989)] For both ob and db, congenic C57BL/Ks mice develop a severe diabetes with ultimate β cell necrosis and islet atrophy, resulting in a relative insulinopenia. Conversely, congenic C57BL/6J ob and db mice develop a transient insulin-resistant diabetes that is eventually compensated by β cell by hypertrophy resembling human Type II diabetes.

The phenotype of ob and db mice resembles human obesity in ways other than the development of diabetes - the mutant mice eat more and expend less energy than do lean controls (as do obese humans). This phenotype is also quite similar to that seen in animals with lesions of the ventromedial hypothalamus, which suggests that both mutations may interfere with the ability to properly integrate or respond to nutritional information within the central nervous system. Support for this hypothesis comes from the results of parabiosis experiments [Coleman, Diabetologia, 9:294-298 (1973)] that suggest ob mice are deficient in a circulating satiety factor and that db mice are resistant to the effects of the ob factor (possibly due to an ob receptor defect). These experiments have led to the conclusion that obesity in these mutant mice may result from different defects in an afferent loop and/or integrative center of the postulated feedback mechanism that controls body composition

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Using molecular and classical genetic markers, the *ob* and *db* genes have been mapped to proximal chromosome 6 and midchromosome 4, respectively [Bahary *et al.*, *Proc. Nat. Acad. Sci. USA*, 87:8642-8646 (1990); Friedman *et al.*, *Genomics*, 11:1054-1062 (1991)]. In both cases, the mutations map to regions of the mouse genome that are syntenic with human, suggesting that, if there are human homologs of *ob* and *db*, they are likely to map, respectively, to human chromosomes 7q and 1p. Defects in the *db* gene may result in obesity in other mammalian species: in genetic crosses between Zucker *fa/fa* rats and Brown Norway +/+ rats, the *fa* mutation (rat chromosome 5) is flanked by the same loci that flank *db* in mouse [Truett *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7806-7809 (1991)]

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Because of the myriad factors that seem to impact body weight, it has not been possible to predict which factors and, more particularly, which homeostatic mechanisms, are primarily determinative of body weight. Thus, the principal problem underlying the present invention is to provide modulators of body weight which allow the control of adiposity and fat content of mammals.

SUMMARY OF THE INVENTION

According to the present invention the problem of control of adiposity and fat content of animals, particularly mammals, has been solved through the provision of obesity (OB) polypeptides and nucleic acid molecules coding for these polypeptides as disclosed herein. The present invention provides, for the first time, isolated polypeptides useful for modulation, i.e., control and regulation, of body weight and adiposity as well as nucleic acid sequences encoding such polypeptides which not only allow for recombinant production of the OB polypeptides but are themselves useful in modulation of body weight.

15 Obesity (OB) polypeptides of the present invention have about 145 to about 167 amino acids, are capable of modulating body weight in an animal, particularly a mammal, and include allelic variants or analogs, including fragments, thereof having the same biological activity. The polypeptides can be prepared by recombinant or chemical synthetic methods. Presently preferred OB polypeptides include those having the amino acid sequence of SEQ ID NOS: 2, 4, 5 or 6, or allelic variants or analogs, including fragments, thereof.

Immunogenic fragments of OB polypeptides of the invention include: Val-Pro-Ile-Gln-Lys-Val-Gln-Asp-Asp-Thr-Lys-Thr-Leu-Ile-Lys-Thr (SEQ ID NO: 18); Leu-His-Pro-Ile-Leu-Ser-Leu-Ser-Lys-Met-Asp-Gln-Thr-Leu-Ala (SEQ ID NO: 19); Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gln-Thr-Ser-Gly-Leu-Gln-Lys-Pro-Glu-Ser-Leu-Asp (SEQ ID NO: 20); and Ser-Arg-Leu-Gln-Gly-Ser-Leu-Gln-Asp-Ile-Leu-Gin-Gin-Leu-Asp-Val-Ser-Pro-Glu-Cys (SEQ ID NO: 21).

Human OB polypeptide analogs include those having the human amino acid sequences of SEQ ID NOS: 4 and 6, wherein one or more of amino acids 53, 56, 71, 85, 89, 92, 95, 98, 110, 118, 121, 122, 126, 127, 128, 129, 132, 139, 157, 159, 163, and 166 (according to the numbering of SEQ ID NO: 4) is substituted with another amino acid such as the divergent amino acid of the mouse OB polypeptide as set out in SEQ ID NO: 2, or an alanine. Such analogs also include those wherein: (a) the serine residue at position 53 is substituted with glycine, alanine, valine, cysteine, methionine, or threonine; (b) the serine residue at position 98 is substituted with glycine, alanine, valine, cysteine, methionine, or threonine; and (c) the arginine residue at position number 92 is substituted with asparagine, lysine, histidine, glutamine, glutamic acid, aspartic acid, serine, threonine, methionine, or cysteine. An OB polypeptide analog according to the invention preferably has 83 percent or greater amino acid sequence homology to the human OB polypeptide amino acid sequence set out in SEQ ID NO: 2, 4, 5 or 6.

- Additional human OB polypeptide analogs according to the invention have the amino acid sequence of SEQ ID NOS: 4 and 6 and have: (a) one or more aspartic acid residues substituted with glutamic acid; (b) one or more isoleucine residues substituted with leucine; (c) one or more glycine or valine residues substituted with alanine; (d) one or more arginine residues substituted with histidine; (e) one or more tyrosine or phenylalanine residues substituted with tryptophan; (f) one or more of residues 121 through 128 (according to the numbering of SEQ ID NO:4) substituted with glycine or alanine; and (g) one or more residues at positions 54 through 60 or 118 through 166 (according to the number of SEQ ID NO: 4) substituted with lysine, glutamic acid, cysteine, or proline.
- 25 Presently preferred human OB polypeptide truncated analogs according to the invention include those wherein (according to the numbering of SEQ ID NO: 4): (a) one or more residues at positions 121 to 128 are deleted; (b) residues 1-116 are deleted; (c) residues 1-21 and 54 to 167 are deleted; (d) residues 1-60 and 117 to 167 are deleted; (e) residues 1-60 are deleted; (f) residues 1-53 are deleted; and, (g) an

analog of subpart (a) wherein residues 1-21 are deleted. OB polypeptides and ob polypeptide analogs of the invention which lack the 21 amino acid "signal" sequence (e.g., amino acids 1 through 21 of SEQ ID NO: 4)can have an N-terminal amino acid or amino acid sequence such as (1) methionine, (2) a glycine-serine-histidine-methionine sequence (SEQ ID NO: 38), (3) a methionine-glycine-serine-histidine-histidine-histidine-histidine-histidine-serine-serine-serine-serine-poline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98), (4) a leucine-glutamic acid-lysine-arginine-glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 26), (5) a glutamic acid-alanine-glutamic acid-alanine (SEQ ID NO: 27), (6) a leucine-glutamic acid-lysine-arginine sequence (SEQ ID NO: 28); (7) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-proline sequence (SEQ ID NO: 99), and (8) a glycine-serine-proline sequence.

15 Derivatives of an OB polypeptide according to the invention have one or more chemical moieties attached thereto including water-soluble polymers such as polyethylene glycol. Polyethylene glycol derivatized derivatives can be mono-, di-, tri- or tetrapegylated ε.g., N-terminal monopegylated. Preferred N-terminal monopegylated derivatives of ob polypeptides of the invention include OB polypeptides comprising the amino acid residues 22 through 167 of SEQ ID NO: 6, optionally having a (pegylated) methionine at position 21.

Isolated nucleic acid molecule provided by the present invention encode an OB polypeptide, allelic variant, or analog, including fragments, as described above.

Specifically provided are DNA molecules for use in securing expression of an OB polypeptide having the biological activity of modulating body weight in a mammal, and selected from the group consisting of: (a) the DNA molecules set out in SEQ ID NOS: I and 3 or fragments thereof; (b) DNA molecules which hybridize to the DNA molecules defined in (a) or hybridizable fragments thereof; and (c) DNA molecules

that code on expression for the amino acid sequence encoded by any of the foregoing DNA molecules. Illustrative of such molecules is the human genomic DNA molecule of SEO ID NOS: 22 and 24.

Preferred DNA molecules according to the invention encode a polypeptide having an amino acid sequence as set out in: (a) SEQ ID NO: 2; (b) amino acids 22 through 167 of SEQ ID NO: 2; (c) SEQ ID NO: 4; (d) amino acids 22 through 167 of SEQ ID NO: 5; (f) amino acids 22 through 166 of SEQ ID NO: 5; and (g) SEQ ID NO: 6; and (h) amino acids 22 through 166 of SEQ ID NO: 6, as well as polypeptides which have an N-terminal amino acid or amino acid sequence as 10 previously noted. Illustratively, a preferred DNA molecule has the sequence set out as the protein coding sequence of SEQ ID NO: 3 and particularly has the sequence set out as the sequence encoding amino acids 22 through 167.

Detectably labeled nucleic acid molecules hybridizable to a DNA molecule of the invention are also provided and include nucleic acid molecules hybridizable to a non-coding region of an OB nucleic acid, which non-coding region is selected from the group consisting of an intron, a 5 ' non-coding region, and a 3 ' non-coding region.

The present invention also provides oligonucleotide primers for amplifying human genomic DNA encoding an ob polypeptide such as oligonucleotides set out in SEQ ID NOS: 29 through 32.

Vectors provided by the invention comprise a DNA molecule according to the invention as described above and preferably have the form of an expression vector which comprises the DNA molecule to operatively associated with an expression control sequence. Unicellular host cells of the invention are transformed or transfected with a DNA molecules of the invention or with a vector as described above. Preferred host cells include bacteria, yeast, mammalian cells, plant cells, insect cells, and human cells in tissue culture. Illustratively, such host cells are selected from the group consisting of E. coli, Pseudomonas, Bacillus, Streptomyces, yeast, CHO, Rl.1, B-W, L-M, COS 1. COS 7, BSC1, BSC40, BMT10, and Sf9

cells. Presently preferred yeast hosts include Saccharomyces, Pichia, Candida, Hansenula and Torulopsis. Also provided are mammalian cells containing an ob polypeptide encoding DNA sequence and modified in vitro to permit higher expression of ob polypeptide by means of a homologous recombinational event consisting of inserting an expression regulatory sequence in functional proximity to the ob polypeptide encoding sequence. The expression regulatory sequence can be an ob polypeptide expression or not and can replace a mutant ob polypeptide regulatory sequence in the cell.

The present invention provides methods for preparing an ob polypeptide comprising:

(a) culturing a cell as described above under conditions that provide for expression of the ob polypeptide; and (b) recovering the expressed ob polypeptide. This procedure can also be accompanied by the steps of: (c) chromatographing the polypeptide on a Ni-chelation column; and (d) purifying the polypeptide by gel filtration. In a preferred embodiment, after step (c) and before step (d), the method includes chromatographing the ob polypeptide on a strong cation exchanger column.

The present invention also provides labeled and unlabeled monoclonal and polyclonal antibodies specific for ob polypeptides of the invention and immortal cell lines that produce a monoclonal antibody of the invention. Antibody preparation according to the invention involves: (a) conjugating an ob polypeptide to a carrier protein; (b) immunizing a host animal with the OB polypeptide fragment-carrier protein conjugate of step (a) admixed with an adjuvant; and (c) obtaining antibody from the immunized host animal.

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The invention provides methods for measuring the presence of an OB polypeptide in a sample, comprising: (a) contacting a sample suspected of containing an OB polypeptide with an antibody (preferably bound to a solid support) that specifically binds to the OB polypeptide under conditions which allow for the formation of reaction complexes comprising the antibody and the OB polypeptide; and (b) detecting

the formation of reaction complexes comprising the antibody and ob polypeptide in the sample, wherein detection of the formation of reaction complexes indicates the presence of OB polypeptide in the sample. Correspondingly provided are in vitro methods for evaluating the level of OB polypeptide in a biological sample comprising: (a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and (b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of OB polypeptide in the biological sample. When detecting or diagnosing the presence of a disease associated with elevated or decreased levels of OB polypeptide according to the invention, an evaluation as above is made and the level detected is compared to a level of OB polypeptide present in normal subjects or in the subject at an earlier time. An increase in the level of OB polypeptide as compared to normal or prior levels indicates a disease associated with elevated levels of OB polypeptide and a decreased level of OB polypeptide as compared to normal levels indicates a disease associated 15 with decreased levels of OB polypeptide. Correspondingly provided are in vitro methods for monitoring therapeutic treatment of a disease associated with elevated or decreased levels of OB polypeptide in a mammalian subject comprising evaluating, as describe above, the levels of OB polypeptide in a series of biological samples obtained at different time points from a mammalian subject undergoing such therapeutic treatment.

Pharmaceutical compositions according to the invention comprise an OB polypeptide as described above together with a pharmaceutically acceptable carrier and are useful in therapeutic methods for reducing the body weight of an animal. Additional pharmaceutical compositions of the invention for use in therapeutic methods for increasing the body weight of an animal comprise an antagonist of an OB polypeptide, preferably selected from the group consisting of an antibody that binds to and neutralizes the activity of the OB polypeptide, a fragment of the ob polypeptide that binds to but does not activate the OB polypeptide receptor, and a small molecule antagonist of the OB polypeptide. The present invention also provides corresponding body appearance improving cosmetic compositions for reducing or increasing the

body weight of an individual, which compositions are useful in cosmetic processes for improving the body appearance of an individual. Such cosmetic compositions are administered to the individual in a dose amount sufficient to modulate the individual's body weight to a desired level.

5 Also addressed by the present invention is the use of nucleic acid moles of the invention, as well as antisense nucleic acid molecules hybridizable to a nucleic acid encoding an OB polypeptide according to the invention, for manufacture of a medicament for (e.g., gene therapy) modification body weight of an animal. Also provided is the use of an OB polypeptide or antagonist according to the invention for the manufacture of a medicament for modification of the body weight of an animal. Medicaments so developed can be employed for modification of the body weight of a mammal in treating a disorder selected from the group consisting of diabetes, high blood pressure and high cholesterol and as part of combinative therapy with a medicament for treating such disorders. Such medicaments can be employed in therapeutic methods involving intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, nasal, oral or pulmonary delivery systems.

Data presented herein show that the establish that the OB polypeptides of the invention in their form are secreted primarily from mammalian adipocytes and that the polypeptides function as hormones.

20 The Examples herein demonstrate that the OB polypeptide, alternatively termed herein "leptin," circulates in mouse, rat, and human plasma. Leptin is absent in plasma from ob/ob mice, and is present at ten-fold higher concentrations in plasma from db/db mice. and twenty-fold higher concentrations in fal/a rats. Most significantly, daily injections of recombinant leptin dramatically reduces the body mass of ob/ob mice, significantly affects the body weight of wild-type mice, and has no effect on db/db mice.

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In a further aspect, the ob polypeptide from one species is biologically active in another species. In particular, the human OB polypeptide is active in mice.

In a first instance, the modulators of the present invention comprise nucleic acid molecules, including recombinant DNA molecules (e.g., cDNA or a vector containing 5 the cDNA or isolated genomic DNA) or cloned genes (i.e., isolated genomic DNA), or degenerate variants thereof, which encode polypeptides themselves serving as modulators of weight control as hereinafter defined, or conserved variants or fragments thereof, particularly such fragments lacking the signal peptide (alternatively referred to herein as mature OB polypeptide), which polypeptides possess amino acid sequences such as set forth in FIGURE 1A through E (SEQ ID NO:2), FIGURE 3 (SEQ ID NO:4), FIGURE 5 (SEQ ID NO:5) and FIGURE 6 (SEQ ID NO:6). In specific embodiments, amino acid sequences for two variants of murine and human ob polypeptides are provided. Both polypeptides are found in a form with glutamine 49 deleted, which may result from an mRNA splicing anomaly. The OB polypeptides from various species may be highly homologous; as shown in Figure 4, murine and human OB polypeptides are greater than 80% homologous.

The nucleic acid molecules, recombinant DNA molecules, or cloned genes, may have the nucleotide sequences or may be complementary to DNA coding sequences shown in FIGURE 1A through E (SEQ ID NO:1) and FIGURE 2A and B (SEQ ID NO:3).

20 In particular, such DNA molecules can be cDNA or genomic DNA isolated from the chromosome. Nucleic acid molecules of the invention may also correspond to 5' and 3' flanking sequences of the DNA and intronic DNA sequences. Accordingly, the present invention also relates to the identification of a nucleic acid having a nucleotide sequence selected from the sequences of Figure 1A through E (SEQ ID NO:1) and Figure 2A and B (SEQ ID NO:3) herein, and degenerate variants, allelic variations.

and like cognate molecules.

A nucleic acid molecule of the invention can be DNA or RNA, including synthetic variants thereof having phosphate or phosphate analog, e.g., thiophosphate, bonds. Both single-stranded and double-stranded sequences are contemplated herein.

The present invention further provides nucleic acid molecules for use as molecular 5 probes, or as primers for polymerase chain reaction (PCR) amplification. i.e., synthetic or natural oligonucleotides having a sequence corresponding to a portion of the sequences shown in Figure 1A through E (SEQ ID NO:1), Figure 2A and B (SEQ ID NO:3) and Figure 20A through C (SEQ ID NOs:22 and 24); or the 5' and 3' flanking sequences of the coding sequences; or intronic sequences of the genomic 10 DNA. In particular, the invention contemplates a nucleic acid molecule having at least about 10 nucleotides, wherein a sequence of the nucleic acid molecule corresponds to a nucleotide sequence of the same number of nucleotides in the nucleotide sequences of Figure 1A through E (SEQ ID NO:1), Figure 2A and B (SEQ ID NO:3) and Figure 20A through C (SEQ ID NO:22), or a sequence complementary 15 thereto. More preferably, the nucleic acid sequence of the molecule has at least 15 nucleotides. Most preferably, the nucleic acid sequence has at least 20 nucleotides. In an embodiment of the invention in which the oligonucleotide is a probe, the oligonucleotide is detectably labeled, e.g., with a radionuclide (such as 32P), or an enzyme.

20 In further aspects, the present invention provides a cloning vector, which comprises the nucleic acids of the invention that encode the ob polypeptide; and a bacterial, insect, or a mammalian expression vector, which comprises the nucleic acid molecules of the invention encoding the ob polypeptide, operatively associated with an expression control sequence. Accordingly, the invention further relates to a host cell, such as a bacterial cell, yeast cell, insect cell, or a mammalian cell, transfected or transformed with an appropriate expression vector, and correspondingly, to the use of the above mentioned constructs in the preparation of the modulators of the invention.

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In yet a further aspect, the present invention relates to antibodies that bind to the ob polypeptide. Such antibodies may be generated against the full-length polypeptide, or antigenic fragments thereof. In one aspect, such antibodies inhibit the functional (i.e., body weight and fat composition modulating) activity of the ob polypeptide.

In another aspect, antibodies can be used to determine the level of circulating ob polypeptide in plasma or serum. In yet a further aspect, region-specific antibodies, particularly monoclonal antibodies, can be used as probes of OB polypeptide structure.

All of the foregoing materials are to be considered herein as modulators of body
weight and fat composition, and as such, may be used in a variety of contexts.
Specifically, the invention contemplates both diagnostic and therapeutic applications,
as well as certain agricultural applications, all contingent upon the use of the
modulators defined herein, including both nucleic acid molecules and peptides.
Moreover, the modulation of body weight carries specific therapeutic implications and
benefits, in that conditions where either obesity or, conversely, cachexia represent
undesired bodily conditions, can be remedied by the administration of one or more
of the modulators of the present invention.

Thus, a method for modulating body weight of a mammal is proposed that comprises controlling the expression of the protein encoded by a nucleic acid having a nucleotide sequence selected from the sequence of Figure 1A through E (SEQ ID NO:1), the sequence of Figure 2A and B (SEQ ID NO:3) and degenerate and allelic variants thereof. Such control may be effected by the introduction of the nucleotides in question by gene therapy into fat cells of the patient or host to control or reduce obesity. Conversely, the preparation and administration of antagonists to the nucleotides, such as anti-sense molecules, would be indicated and pursued in the instance where conditions involving excessive weight loss, such as anti-such nervosa, cancer, or AIDS are present and under treatment. Such constructs would be introduced in a similar fashion to the nucleotides, directly into fat cells to effect such changes.

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Correspondingly, the proteins defined by Figures 1A through E, 3, 5, and 6 (SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6), conserved variants, active fragments thereof, and cognate small molecules could be formulated for direct administration for therapeutic purposes, to effect reduction or control of excessive 5 body fat or weight gain. Correspondingly, antibodies and other antagonists to the stated protein materials, such as fragments thereof, could be prepared and similarly Accordingly, the invention is administered to achieve the converse effect. advantageously directed to a pharmaceutical composition comprising an OB polypeptide of the invention, or alternatively an antagonist thereof, in an admixture with a pharmaceutically acceptable carrier or excipient.

In addition, the OB polypeptide of the invention may be administered for its cosmetic effects, e.g., to improve body appearance by reducing fat deposits. The OB polypeptide can be used independently or in conjunction with other cosmetic strategies, e.g., surgery, for its cosmetic effects.

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15 The diagnostic uses of the present nucleotides and corresponding peptides extend to the use of the nucleic acids to identify further mutations of allelic variations thereof, so as to develop a repertoire of active nucleotide materials useful in both diagnostic and therapeutic applications. In particular, both homozygous and heterozygous mutations of the nucleotides in question could be identified that would be postulated to more precisely quantitate the condition of patients, to determine the at-risk potential of individuals with regard to obesity. Specifically, heterozygous mutations are presently viewed as associated with mild to moderate obesity, while homozygous mutations would be associated with a more pronounced and severe obese condition. Corresponding DNA testing could then be conducted utilizing the aforementioned ascertained materials as benchmarks, to facilitate an accurate long term prognosis for particular tendencies, so as to be able to prescribe changes in either dietary or other personal habits, or direct therapeutic intervention, to avert such conditions.

The diagnostic utility of the present invention extends to methods for measuring the presence and extent of the modulators of the invention in cellular samples or biological extracts (or samples) taken from test subjects, so that both the nucleic acids (genomic DNA or mRNA) and/or the levels of protein in such test samples could be ascertained. Given that the increased activity of the nucleotide and presence of the resulting protein reflect the capability of the subject to inhibit obesity, the physician reviewing such results in an obese subject would determine that a factor other than dysfunction with respect to the presence and activity of the nucleotides of the present invention is a cause of the obese condition. Conversely, depressed levels of the nucleotide and/or the expressed protein would suggest that such levels must be increased to treat such obese condition, and an appropriate therapeutic regimen could then be implemented.

Further, the nucleotides discovered and presented in Figures 1A through E and 2A and B represent cDNA which, as stated briefly above, is useful in the measurement of corresponding RNA. Likewise, recombinant protein material corresponding to the polypeptides of Figures 1A through E and 3 may be prepared and appropriately labeled, for use, for example, in radioimmunoassays, for example, for the purpose of measuring fat and/or plasma levels of the OB protein, or for detecting the presence and level of a receptor for OB on tissues, such as the hypothalamus.

Yet further, the present invention contemplates not only the identification of the nucleotides and corresponding proteins presented herein, but the elucidation of the receptor to such materials. In such context, the polypeptides of Figures 1A through E, 3, 5, and/or 6 could be prepared and utilized to screen an appropriate expression library to isolate active receptors. The receptor could thereafter be cloned, and the receptor alone or in conjunction with the ligand could thereafter be utilized to screen for small molecules that may possess like activity to the modulators herein.

Yet further, the present invention relates to pharmaceutical compositions that include certain of the modulators hereof, preferably the polypeptides whose sequences are WO 96/05309 PCT/US95/10479

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presented in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, their antibodies, corresponding small molecule agonists or antagonists thereof, or active fragments prepared in formulations for a variety of modes of administration, where such therapy is appropriate. Such formulations would include pharmaceutically acceptable carriers, or other adjuvants as needed, and would be prepared in effective dosage ranges to be determined by the clinician or the physician in each instance.

Accordingly, it is a principal object of the present invention to provide modulators of body weight as defined herein in purified form, that exhibit certain characteristics and activities associated with control and variation of adiposity and fat content of

It is a further object of the present invention to provide methods for the detection and measurement of the modulators of weight control as set forth herein, as a means of the effective diagnosis and monitoring of pathological conditions wherein the variation in level of such modulators is or may be a characterizing feature.

15 It is a still further object of the present invention to provide a method and associated assay system for the screening of substances, such as drugs, agents and the like, that are potentially effective to either mimic or inhibit the activity of the modulators of the invention in mammals.

It is a still further object of the present invention to provide a method for the treatment of mammals to control body weight and fat content in mammals, and/or to treat certain of the pathological conditions of which abnormal depression or elevation of body weight is a characterizing feature.

It is a still further object of the present invention to prepare genetic constructs for use in genetic therapeutic protocols and/or pharmaceutical compositions for comparable therapeutic methods, which comprise or are based upon one or more of the

modulators, binding partners, or agents that may control their production, or that may mimic or antagonize their activities.

Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 (A through E) depicts the nucleic acid sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) derived for the murine OB cDNA. A 39 base pair 5' leader was followed by a predicted 167 amino acid open reading frame and an approximately 3.7 kb 3' untranslated sequence. (In previously filed application Serial No. 08/347,563 filed November 30, 1994 and Serial No. 08/438,431, filed May 10, 1995, an additional 58-base 5' non-coding sequence was determined subsequently, to be a cloning artifact. This artifact has no bearing on the coding region, the 39 base 5' non-coding region presently depicted in FIGURE 1, or 3' non-coding region of the gene.) A total of about 2500 base pairs of the 3' untranslated sequence is shown. Analysis of the predicted protein sequence by observation and using the SigSeq computer program indicates the presence of a signal sequence (underlined). Microheterogeneity of the cDNA was noted in that approximately 70% of the cDNAs had a glutamine codon at codon 49 and 30% did 20 not (see FIGURES 5 and 6, infra). This amino acid is underlined, as is the arginine codon that is mutated in C57BL/6J ob/ob mice (1J mice).

FIGURE 2 (A and B) depicts the nucleic acid sequence (SEQ ID NO:3) derived for the human OB cDNA. The nucleotides are numbered from 1 to 701 with a start site at nucleotide 46 and a termination at nucleotide 550.

25 FIGURE 3 depicts the full deduced amino acid sequence (SEQ ID NO:4) derived for the human OB gene corresponding to the nucleic acid sequence of FIGURE 2A and B. The amino acids are numbered from 1 to 167. A signal sequence cleavage site is located after amino acid 21 (Ala) so that the mature protein extends from amino acid 22 (Val) to amino acid 167 (Cys).

FIGURE 4 depicts the comparison between the murine (SEQ ID NO:2) and human 5 (SEQ ID NO:4) deduced amino acid sequences. The sequence of the human OB deduced amino acid sequence was highly homologous to that of mouse. Conservative changes are noted by a dash, and non-conservative changes by an asterisk. The variable glutamine codon is underlined, as is the position of the nonsense mutation in C57BL/6J ob/ob (1J) mice. Overall, there is 83% identity at the amino acid level, although only eight substitutions were found between the valine at codon 22 (immediately downstream of the signal sequence overage) and the cysteine at position 117.

FIGURE 5 depicts the full length amino acid sequence (SEQ ID NO:5) derived for the murine OB gene as shown in FIGURE 3, but lacking glutamine at position 49. The amino acids are numbered from 1 to 166. A signal sequence cleavage site is located after amino acid 21 (Ala) (and thus, before the glutamine 49 deletion) so that the mature protein extends from amino acid 22 (Val) to amino acid 166 (Cys).

FIGURE 6 depicts the full deduced amino acid sequence (SEQ ID NO:6) derived for the human OB gene as shown in FIGURE 4, but lacking glutamine at position 49.

The amino acids are numbered from 1 to 166. A signal sequence cleavage site is located after amino acid 21 (Ala) (and thus, before the glutamine 49 deletion) so that the mature protein extends from amino acid 22 (Val) to amino acid 166 (Cys).

FIGURE 7. (A) Physical map of the location of *ob* in the murine chromosome, and the YAC and P1 cloning maps. "M and N" corresponds to *Mul*1 and *Not*1 restriction sizes. The numbers correspond to individual animals that were recombinant in the region of *ob* of the 1606 meioses that were scored. Met, Pax 4, D6Rck39, D6Rck13, and Cpa refer to locations in the region of *ob* that bind to the DNA probes. YACs

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were isolated using D6Rck13 and Pax-4 as probes, and the ends were recovered using vectorette PCR and/or plasmid end rescue and used in turn to isolate new YACs. (B)

The resulting YAC contig. One of the YACs in this contig, Y902A0925, was chimeric. Each of the probes used to genotype the recombinant animals is indicated in parentheses. (6) Corresponds to YAC 107; (5) corresponds to M16(+) (or M16(pLUS)); (4) corresponds to adu(+); (3) corresponds to aad(pICL); (2) corresponds to 53(pICL); and (1) corresponds to 53(+). (C) The P1 contig of bacteriophage P1 clones isolated with selected YAC end probes. The ob gene was isolated in a P1 clone isolated using the distal end of YAC YB6S2F12 (end (4))

10 (alternatively termed herein adu(+)).

FIGURE 8 presents a photograph of an ethidium bromide stain of 192 independent isolates of the fourth exon trapping experiment that were PCR amplified and characterized

FIGURE 9 is a photograph of an ethidium bromide stain of PCR-amplified clones suspected of carrying ob. Each of the 7 clones that did not carry the artifact was reamplified using PCR and electrophoresed on a 1% agarose gel in TBE and stained with ethidium bromide. The size markers (far left unnumbered lane) are the commercially available "1 kB ladder". Lane 1 -- clone 1D12, containing an "HIV sequence." Lane 2 -- clone 1F1, a novel clone outside of the ob region. Lane 3 -- clone 1H3. Lane 4 -- clone 2B2, which is the identical to 1F1. Lane 5 -- clone 2G7, which contains an ob exon. Lane 6 -- clone 2G11, which is identical to 1F1. Lane 7 -- clone 2H1, which does not contain an insert.

FIGURE 10 presents the sequence of the 2G7 clone (SEQ ID NO:7), which includes an exon coding for a part of the OB gene. The primer sequences used to amplify this exon are boxed in the figure (SEO ID NOS:8 and 9).

FIGURE 11 (A) Reverse transcription-PCR analysis of mRNA from different tissues of the same mouse with the 2G7 primers and actin primers. The RT-PCR reactions were performed using 100 ng of total RNA reverse transcribed with oligo dT as a primer for first strand cDNA synthesis. PCR amplification was performed for 35 cycles with 94° denaturation for 1'; 55° hybridization for 1'; and 72°C extensions for 2' with a 1' second autoextension per cycle. RT-PCR products were resolved in a 2% low melting point agarose gel run in 1x TBE buffer. (B) Northern blot of mRNA from different organs of the mouse using PCR labeled 2G7 as a probe. Ten µg of total RNA from each of the tissues was electrophoresed on an agarose gel with formaldehyde. The probe was hybridized at 65°C in Rapid Hybe (Amersham). Autoradiographic signals were apparent after 1 hour of exposure; the experiment shown was the result of a 24 hour exposure.

- FIGURE 12 (A) An ethidium bromide stain from an RT-PCR reaction on fat cell (white adipose tissue) RNA from each of the mouse strains listed. Total RNA (100 ng) for each sample was reverse transcribed using oligo dT and reverse transcriptase, and the resulting single-stranded cDNA was PCR amplified with the 2G7 primers (lower bands) or actin primers (upper bands). Both the 2G7 and actin primers were included in the same PCR reaction. The products were run on a 1% agarose TBE gel. (B) Northern analysis corresponding to (A). Ten µg of fat cell (white adipose tissue) RNA from each of the strains indicated were run out and probed with the PCR labeled 2G7 probe as in Figure 11B, above. An approximately 20-fold increase in the level of 2G7 mRNA was apparent in white fat RNA from the C57BL/6J ob/ob (1J) strain relative to lean littermates. In both the RT-PCR and Northern experiments there was no detectable signal in 2G7 RNA from the SM/Ckc-+^{Dwc}ob²⁰/ob²⁰ (2J) mice even after a 2 week exposure. A 24 hour autoradiographic exposure is shown. The same filter was hybridized to an actin probe (bottom portion of the panel).
- 25 FIGURE 13 is a Northern analysis of additional 2J animals and control animals that confirms the absence of the ob mRNA from 2J animals. The Northern analysis was performed as in Figures 11 and 12. In this case, the control RNA was ap2, a fail specific transcript. There is no significance to the varying density of the ap2 bands.

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FIGURE 14 compares the DNA sequence of the C57BL/6J (normal) and the C57BL/6J ob/ob (1J) mice in the region of the point mutation that leads to introduction of a premature stop codon (nonsense mutation) in the mutant strain cDNA. The ob/ob mice had a C+T mutation that changed an arginine residue at position 105. This base change is shown as the output from the automated DNA sequencer. RT-PCR was performed using white fat RNA from both strains (+/+ and ob/ob) using primers from the 5' and 3' untranslated regions. The PCR reaction products were gel purified and directly sequenced manually and using an Applied Biosystems, Inc. 373A automated sequencer with primers along both strands of the ocding sequence.

FIGURE 15 (A) Southern blot of genomic DNA from each of the mouse strains listed. Approximately 5 µg of DNA (derived from genomic DNA prepared from liver, kidney or spleen) was restriction digested with the restriction enzyme indicated. The DNA was then electrophoresed in a 1% agarose TBE gel and probed with PCR labeled 2G7. Restriction digestion with Bg/III revealed an increase in the size of an approximately 9 kB (the largest) Bg/III fragment in SM/Ckc-+\(^{\text{Dac}}\)ob^2/(\(^{\text{D}}\)')\(^{\text{D}}\)' (2J) DNA. RFLPs were not detectable with any other restriction enzymes. Preliminary restriction mapping of genomic DNA indicated that the polymorphic Bg/III site is about 7 kB upstream of the transcription start site. None of the other enzymes tested extend past the mRNA start site. (B) Segregation of a Bg/III polymorphism in the SM/Ckc-+\(^{\text{Dac}}\)ob^2/\(^{\text{D}}\)'\(^{\text{D}}\)' ob^2 strain. Six obese and five lean progeny from the same generation of the coisogenic SM/Ckc-+\(^{\text{Dac}}\)ob^2/\(^{\text{D}}\)' (2J) colony were genotyped by scoring the \(^{\text{B}}\)g/III polymorphism as shown in (A). All of the phenotypically obese animals were homozygous for the larger allele of the polymorphic \(^{\text{B}}\)II fragment.

FIGURE 16 is a Southern blot of *LcoRl* digested genomic DNA from the species listed, using an *OB* cDNA as a probe (i.e., a zoo blot). Hybridization signals were detectable in every vertebrate sample, even after a moderate stringency hybridization.

mouse, bred separately from the SM/Ckc-+ $^{Dac}ob^{2l}/ob^{2l}$ colony.

The cat DNA in this experiment was slightly degraded. The restricted DNA was run on a 1% agarose TBE gel, and transferred to an imobilion membrane for probing. The filter was hybridized at 65°C and washed in 2X SSC/0.2% SDS at 65°C twice for twenty minutes and exposed for 3 days using Kodak (Rochester, N.Y.) X-OMAT film.

FIGURE 17 presents the expression cloning region of vector pET-15b (Novagen) (SEO ID NOS.: 11 and 12).

FIGURE 18 presents analysis of the eluate from a His-binding resin (Ni) column for a recombinant mature murine ob fusion to a His-tag (A) and mature human OB fusion to a His-tag (B). Bacteria were transformed with vectors pETM9 and pETH14, respectively. Upon induction with 1 mM IPTG at optimal conditions, the transformed bacteria were able to produce 100-300 μg/ml of OB fusion protein, primarily in the inclusion bodies. The inclusion bodies were solubilized with 6M guanidine-HCl or urea, and fusion protein (present in the lysis supernatant) was loaded on the Hisbinding resin (Ni) column in 10 ml of 1x binding buffer with urea. The column was eluted stepwise with 5 ml aliquots of 20 μM, 60 μM, and 300 μM imidazole, and finally with strip buffer. The aliquots were analyzed for the presence of OB polypeptide fusion on a 15% acrylamide gel. Each lane contains the equivalent of 100 μl of bacterial extract.

20 FIGURE 19 (A) In vitro translation of OB RNA. A human OB cDNA was subcloned into the pGEM vector. The plasmid was linearized and plus strand RNA was synthesized using Sp6 polymerase. The in vitro synthesized RNA was translated in the presence or absence of canine pancreatic microsomal membranes. An approximately 18 kD primary translation product was seen after in vitro translation.
25 The addition of microsomal membranes to the reaction led to the appearance of a second translation product about 2 kD smaller than the primary translation product. The size of the translation product of interleukin-lα RNA, which lacks an encoded signal sequence, was unchanged by the addition of microsomal membranes. These

data indicated the presence of a functional signal sequence. (B) In vitro translation in the presence or absence of proteinase K. Protease treatment resulted in complete proteolysis of the 18 kD primary translation product, while the 16 kD processed form was unaffected. Permeabilization of the microsome with 0.1% TRITON-X100 rendered the processed form protease sensitive. These results indicate that the product had translated into the lumen of the microsome.

FIGURE 20 (A through E) The sequence of the human OB gene (SEQ ID NOs:22 and 24). (F) A schematic diagram of the murine OB gene. (G) A schematic diagram of the human OB gene. In both (F) and (G), the start and stop codons are underlined.

10 There is no evidence of a first intron homologous to the mouse first intron in the human gene, but its existence cannot be excluded.

FIGURE 21 presents a schematic drawing of one of the cloning strategies employed to achieve recombinant expression of OB in Pichia yeast. (A) Expression vector of OB with an α-mating factor signal sequence. (B) Schematic drawing of the structure of the recombinant fusion protein, including the amino acid sequence (SEQ ID NO:26) showing the XhoI site and putative KEX-2 and STE-13 cleavage sites, and the N-terminal surplus amino acids present after KEX-2 cleavage (SEQ ID NO:27). (C) An alternative strategy for producing mature OB involves preparing a construct with an amino acid sequence corresponding to a XhoI cleavage site and a KEX-2 cleavage site immediately upstream of the mature ob polypeptide sequence (SEQ ID NO:28).

FIGURE 22 Alternative expression strategy in Pichia. (A) Expression vector of an OB fusion with a His-tag adopted from the pET expression system under control of the α -mating factor signal sequence (SEQ ID NO:33). (B) Schematic drawing of the structure of the recombinant OB fusion protein containing a His-tag, which includes the α -mating factor signal sequence, putative KEX-2 and STE-13 cleavage sites, the His-tag, and a thrombin cleavage site, which would yield OB with three surplus N-terminal amino acid residues

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(A)

FIGURE 23 (A) PAGE analysis of expression of murine OB (both the microheterogenous forms, i.e., containing and missing Gln 49) in transformed pichia yeast. The expected band of approximately 16 kD is visible in the transformed yeast culture fluid (second and third lanes), but not in culture fluid from non-transformed 5 yeast (first lane). (B) PAGE analysis of partially purified recombinant OB polypeptide on carboxymethyl cellulose, a weak cation exchanger. A band of about 16 kD is very visible in fractions 3 and 4 from the column, which was eluted with 250 mM NaCl. Lane 1 -- loaded sample; lane 2 - flow through; lanes 3-5 -fractions eluted with 250 mM NaCl.

10 FIGURE 24 shows that the OB protein circulates in mouse plasma. Immunoprecipitations from mouse blood. 0.5 ml of mouse plasma was pre-cleared with unconjugated sepharose and incubated overnight with immunopurified anti-OB antibodies conjugated to sepharose 4B beads. The immunoprecipitate was separated on a 15% SDS-PAGE gel, transferred and Western blotted with an anti-OB antibody. 15 The protein migrated with a molecular weight of approximately 16 kD, to the same position as the mature mouse ob protein expressed in yeast. The protein was absent in plasma from C57BL/6J ob/ob mice and increased ten-fold in plasma from C57BLB/Ks db/db mice relative to wild type mice. db mice have been suggested to overproduce the OB protein, secondary to resistance to its effects. (B) Increased 20 levels of OB in fatty rats. The fatty rat is obese as a result of a recessive mutation on rat chromosome 5. Genetic data has suggested a defect in the same gene mutated in db mice. Plasma from fatty rats and lean littermates was immunoprecipitated and run on Western blots. A twenty-fold increase in the circulating level of OB is seen in the mutant animals. (C). Quantitation of the OB protein in mouse plasma. Increasing amounts of the recombinant mouse protein were added to $100\ \lambda$ of plasma from ob mice and immunoprecipitated. The signal intensity on Western blots was compared to that from 100 \(\lambda\) of plasma from wild-type mice. A linear increase in signal intensity was seen with increasing amounts of recombinant protein demonstrating that the immunoprecipitations were performed under conditions of 30 antibody excess. Similar signals were seen in the wild-type plasma sample and the

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sample with 2 ng of recombinant protein indicating the circulating level in mouse plasma is approximately 20 ng/ml. (D) OB protein in adipose tissue extracts. Cytoplasmic extracts of mouse adipose tissue were prepared from db and wild-type mice. Western blots showed increased levels of the 16 kD protein in extracts prepared from db mice.

FIGURE 25 shows that the OB protein circulates at variable levels in human plasma. (A) Western blots of human plasma. Plasma samples were obtained from six lean volunteers. Immunoprecipitation and Western blotting revealed the presence of an immunoreactive 16 kD protein, identical in size to a recombinant 146 amino acid human protein expressed in yeast. Variable levels of the protein were seen in each of the six samples. (B) An ELISA (Enzyme Linked Immunoassay) for human ob. Microtiter plates were coated with immunopurified anti-human OB antibodies. Known amounts of recombinant protein were added to the plates and detected using immunopurified biotinylated anti-ob antibodies. Absorbance at 414 nm was plotted 15 against known concentrations of OB to yield a standard curve. The resulting standard curve showed that the assay was capable of detecting 1 ng/ml or more of the human OB protein. (C) Quantitation of the OB protein in human plasma. An ELISA immunoassay was performed using 100 \(\lambda\) of plasma from the six lean volunteers and the standards used in panel B. Levels of the OB protein ranging from 2 ng/ml in 20 HP1 to 15 ng/ml in HP6 were seen. These data correlated with the Western blot data in panel A.

FIGURE 26 shows that the OB protein forms inter- or intramolecular disulphide bonds. (A) Western blots under reducing and non-reducing conditions. The Western blots of mouse and human plasma were repeated with and without the addition of reducing agents to the sample buffer. When β-mercaptoethanol is omitted from the sample buffer, immunoprecipitates from db plasma migrate with an apparent molecular mass of 16 kD and 32 kD. Addition of β-mercaptoethanol to the buffer leads to the disappearance of the 32 kD molety (see Figure 24). This result is recapitulated when the mouse protein is expressed in the yeast, *Pichia pastoris*. In

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this case, the mouse OB protein migrates to the position of a dimer. Under reducing conditions the purified recombinant mouse protein migrates with an apparent molecular weight of 16 kD, indicating that the 32 kD molecular form is the result of one or two intermolecular disuphide bonds. The human protein expressed in vivo and 5 in Pichia pastoris migrates with a molecular mass of 16 kD under both reducing and non-reducing conditions (data not shown). (B) The human protein expressed in yeast contains an intramolecular disulphide bond. Secreted proteins generally assume their correct conformation when expressed in the Pichia pastoris expression system. The 146 amino acid mature human protein was expressed in Pichia pastoris and purified from the yeast media by a two-step purification protocol involving IMAC and gel 10 filtration. The purified recombinant protein was subjected to mass spectrometry before and after cyanogen bromide cleavage. Cyanogen bromide cleaves at the carboxy terminus of methionine residues. The molecular mass of the recombinant yeast protein was 16,024±3 Da (calculated molecular mass = 16,024 Da). Cyanogen bromide cleaves after the three methionines in the protein sequence at amino acids 75, 89, and 157. The cyanogen bromide fragment with measured mass 8435.6 Da corresponds to amino acids 90-157 and 158-167 joined by a disulphide linkage between cys-117 and cys-167 (calculated molecular mass = 8434.5 Da). N.D. = note detected.

20 FIGURE 27 depicts the preparation of the bioactive recombinant protein. The nucleotide sequence corresponding to the 145 amino acid mature mouse OB protein was cloned into the pET 15b expression vector. This pET vector inserts a polyhistidine tract (His-tag) upstream of the cloned sequence which allows efficient purification using Immobilized Metal Affinity Chromatography (IMAC). The recombinant bacterial protein initially partitioned in the insoluble membrane fraction after bacterial lysis. The membrane fraction was solubilized using guanidium hydrochloride and loaded onto an IMAC column. The protein was eluted stepwise with increasing concentrations of imidazole as shown. The eluted protein was refolded and treated with thrombin to remove the His-tag, as described below. The

FIGURE 28 shows the biologic effects of the OB protein. Time course of food intake (panels A-C) and body weight (panels D-F). Groups of ten animals received either daily intraperitoneal injections of the OB protein at a dose of 5 mg/kg/day (solid squares), daily injections of PBS (solid circles) or no treatment (solid triangles). 5 The treatment groups included C57Bl/6J ob/ob mice (panels A and D), C57Bl/Ks db/db mice (panels B and E) and CBA/J+/+ mice (panels C and F). The food intake of the mice was measured daily and the body weight was recorded at three to four day intervals as indicated. (The scale of the body weight in grams is different for the wild-type mice vs. the ob and db mice.) The food intake of the ob mice 10 receiving protein was reduced after the first injection and stabilized after the fourth day at a level approximately 40% of that seen in the sham injected group (p < .001). The body weight of these animals decreased an average of 1.3 grams/day and stabilized after three weeks to a level approximately 60% of the starting weight (p < .0001). No effect of the protein was demonstrable in db mice. Small but significant 15 effects on body weight were observed in CBA/J mice at two early time points (p < .02). The standard error of each measure is depicted by a bar and the statistical significance of these results is shown in Table 1.

FIGURE 29 shows the results of pair feeding of ob mice. (A) A group of four C57Bl/6J ob/ob mice were fed an amount of food equal to that consumed by the 20 group of ob mice receiving recombinant protein. The weight loss for both groups was calculated after five, eight, and twelve days. The food-restricted mice lost (hatched bar) less weight than the ob mice receiving protein (solid bar) (p < .02). This result indicates that the weight-reducing effect of the OB protein is the result of effects on both food intake and energy expenditure. (B) Photograph of a treated ob 25 mouse. Shown are two C57Bl/6J ob/ob mice. The mouse on the left received PBS and weighed 65 grams, which was the starting weight. The mouse on the right received daily injections of the recombinant OB protein. The starting weight of this animal was also 65 grams, and the weight after three weeks of protein treatment was 38 grams. (C) Livers from treated and untreated ob mice. Shown are livers from treated and untreated C57Bl/6J ob/ob mice. The liver from the mouse receiving PBS

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had the gross appearance of a fatty liver and weighed 5.04 grams. The liver from the mouse receiving the recombinant ob protein had a normal appearance and weighed 2.23 grams.

Figure 30 shows the *in situ* hybridization of *ob* to adipose tissue. Sense and antisense 5 *ob* RNA was labeled *in vitro* using Sp6 and T7 polymerase and digoxigenin. The labeled RNAs were hybridized to paraffin embedded sections of adipose tissue from epididymal fat pads of eight week old C57Bl/Ks mice (labeled wild-type) and C57Bl/Ks *db/db* mice (labeled *db*). In the figure, the lipid droplets appear as unstained vacuoles within cells. The cytoplasm is a thin rim at the periphery of the cells and is indistinguishable from the cell membrane. Hybridization to all the adipocytes in the field was detected in the wild-type sections only using the antisense probe and greatly increased levels were seen in the tissue sections from the *db/db* animals.

FIGURE 31 shows that OB RNA is expressed in adipocytes in vivo and in vitro.

Total RNA (10 micrograms) from several different sources was electophoresed on blotted and hybridized to an ob probe. Firstly, differences in cell buoyancy after collagenase digestion was used to purify adipocytes. OB RNA was present only in the adipocyte fraction. Lane S indicates the stromovascular fraction and A indicates the adipocyte fraction. In addition, OB RNA was not expressed in the undifferentiated 3T3-442 preadipocyte cells lane U. Differentiated adipocytes from these cell lines expressed clearly detectable levels of OB mRNA (lane D).

FIGURE 32 shows that OB RNA is expressed in all adipose tissue depots. All of the adipose tissue depots tested expressed ob RNA. The inguinal fat pad expressed somewhat lower RNA levels, although there was variability in the level of signals in different experiments. (Figure 31A) Lanes (1) epididymal (2) inguinal (3) abdominal (4) parametrial fat pads. Brown fat also expressed a low level of OB RNA. (Figure 31B) The level of OB expression in brown fat was unchanged in animals housed at

4°C for one week while the abundance of the brown fat specific UCP RNA, known to be cold inducible, increased five-fold.

FIGURE 33 depicts the expression of OB RNA in db/db and gold thioglucose treated mice. Total RNA from the parametrial fat pads of gold thioglucose (GTG) and db/db treated mice was electrophoresed and Northern blotted. GTG administered as a single dose is known to cause obesity by inducing specific hypothalamic lesions. (A) One month old CBA female mice were treated with GTG (.2 mg/g), with a resulting increase of >20 g in treated animals relative to control animals (<5 g). (B) Hybridization of an OB probe to RNA from db/db and GTG treated mice revealed a twenty-fold increase in the abundance of ob RNA relative to control RNA (actin or GAPDH).

FIGURE 34 represents a Northern blot analysis of human RNA. Northern blots containing 10 mg of total RNA from human adipose tissue (FAT, panel A) and 2 mg

of polyA+ RNA from other human tissues (panel B) were hybridized to human ob or human 6-actin probes as indicated. An intense signal at approximately 4.5 kb was seen with the adipose tissue total RNA. Hybridization to the polyA+ RNA revealed detectable signals in heart (HE) and placenta (PL), whereas OB RNA was not detected in brain (BR), lung (LU), liver (LI), skeletal muscle (SM), kidney (KI), and pancreas (PA). In each case, the length of the autoradiographic exposure is indicated. Of note, the genesis of the lower molecular bands seen in placental RNA (e.g., alternate splicing, RNA degradation) is not known.

FIGURE 35 represents YAC contig containing the human OB gene and 8 microsatellite markers. The YAC-based STS-content map of the region of chromosome 7 containing the human OB gene is depicted, as deduced by SEGMAP/Version 3.29 [Green et al., PCR Methods Applic., 1.77-90 (1991)] The 19 uniquely-ordered STSs (see Table 3) are listed along the top. The 8 microsatellite-specific STSs are indicated with stars (see Table 4). Also indicated are the STSs corresponding to the Pax4 and OB genes as well as the predicted positions of the

centromere (CEN) and 7q telomere (TEL) relative to the contig. Each of the 43 YAC clones is depicted by a horizontal bar, with its name given to the left and estimated YAC size (in kb, measured by pulsed-field gel electrophoresis) provided in parenthesis. The presence of an STS in a YAC is indicated by a darkened circle 5 at the appropriate position. When an STS corresponds to the insert end of a YAC, a square is placed around the corresponding circle, both along the top (near the STS name) and at the end of the YAC from which it was derived. For the 5 YACs at the bottom (below the horizontal dashed line), 1 or more STS(s) expected to be present (based on the established STS order) was not detected (as assessed by testing the 10 individual YACs with the corresponding STS-specific PCR assay(s) at least twice), and these are depicted as open circles at the appropriate positions. Most of the YACs were isolated from a human-hamster hybrid cell-derived library [Green et al., Genomics, 25:170-183 (1995)], with their original names as indicated. The remaining YACs were isolated from total human genomic libraries, and their original library 15 locations are provided in Table 3. Boxes are placed around the names of the 3 YACs (vWSS691, vWSS999, and vWSS2935) that were found by FISH analysis to map to 7q31.3. The contig is displayed in its 'uncomputed' form, where YAC sizes are not used to estimate clone overlaps or STS spacing, and all of the STSs are therefore spaced in an equidistant fashion. In the 'computed' form, where YAC sizes are used 20 to estimate the relative distance separating each pair of adjacent STSs as well as the extent of clone overlaps, the total YAC contig appears to span just over 2 Mb.

DETAILED DESCRIPTION

The present invention relates to the elucidation and discovery of a protein, termed herein ob polypeptide or leptin, nucleic acids encoding the protein, including degenerate variations thereof, e.g., that incorporate optimal codons for expression in a particular expression system, which protein demonstrates the ability to participate in the control of mammalian body weight. The nucleic acids in object represent the coding sequences corresponding to the murine and human OB polypeptide, which is postulated to play a critical role in the regulation of body weight and adiposity. Data

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presented herein indicate that the polypeptide product of a nuceic acid of the invention is secreted by the cells that express it, and that the polypeptide functions as a hormone. Additional experimental data demonstrate that the OB polypeptide is very effective in treating obesity in mice carrying a mutation of the *ob* gene. In addition,

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5 high bolus doses or moderate continuous doses of OB polypeptide effect weight reduction in normal (wild-type) mice.

In addition, the Examples herein demonstrate that the OB polypeptide, alternatively termed herein "leptin," circulates in mouse, rat, and human plasma. Leptin is absent in plasma from ob/ob mice, and is present at ten-fold higher concentrations in plasma from db/db mice, and twenty-fold higher concentrations in fa/fa rats. Most significantly, daily injections of recombinant leptin dramatically reduce the body mass of ob/ob mice, significantly affects the body weight of wild-type mice, and has no effect on db/db mice.

In a further aspect, the OB polypeptide from one species is biologically active in another species. In particular, the human OB polypeptide is active in mice.

In its primary aspect, the present invention is directed to the identification of materials that function as modulators of mammalian body weight. In particular, the invention concerns the isolation, purification and sequencing of certain nucleic acids that correspond to the *OB* gene or its coding region in both mice and humans, as well as the corresponding polypeptides expressed by these nucleic acids. The invention thus comprises the discovery of nucleic acids having the nucleotide sequences set forth in FIGURE 1A through E (SEQ ID NO:1) and FIGURE 2A and B (SEQ ID NO:3), and to degenerate variants, alleles and fragments thereof, all possessing the activity of modulating body weight and adiposity. The correspondence of the present nucleic acids to the *OB* gene portends their significant impact on conditions such as obesity as well as other maladies and dysfunctions where abnormalities in body weight are a contributory factor. The invention extends to the proteins expressed by the nucleic acids of the invention, and particularly to those proteins set forth in

FIGURE 1A through E (SEQ ID NO:2), FIGURE 3 (SEQ ID NO:4), FIGURE 5 (SEQ ID NO:5), and FIGURE 6 (SEQ ID NO:6), as well as to conserved variants, active fragments, and cognate small molecules.

As discussed earlier, the weight control modulator peptides or their binding partners or other ligands or agents exhibiting either mimicry or antagonism to them or control over their production, may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient experiencing abnormal fluctuations in body weight or adiposity, either alone or as part of an adverse medical condition such as cancer or AIDS, for the treatment thereof. A variety of administrative techniques may be utilized, among them oral administration, nasal and other forms of transmucosal administration, parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Average quantities of the recognition factors or their subunits may vary and in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian.

In accordance with the above, an assay system for screening potential drugs effective to mimic or antagonize the activity of the weight modulator may be prepared. The weight modulator may be introduced into a test system, and the prospective drug may also be introduced into the resulting cell culture, and the culture thereafter examined to observe any changes in the activity of the cells, due either to the addition of the prospective drug alone, or due to the effect of added quantities of the known weight modulator.

As stated earlier, the molecular cloning of the OB gene described herein has led to the identification of a class of materials that function on the molecular level to modulate mammalian body weight. The discovery of the modulators of the invention has important implications for the diagnosis and treatment of nutritional disorders including, but not limited to, obesity, weight loss associated with cancer and the treatment of diseases associated with obesity such as hypertension, heart disease, and

Type II diabetes. In addition, there are potential agricultural uses for the gene product in cases where one might wish to modulate the body weight of domestic animals. Finally, to the extent that one or more of the modulators of the invention are secreted molecules, they can be used biochemically to isolate their receptor using the technology of expression cloning. The discussion that follows with specific reference to the OB gene bears general applicability to the class of modulators that comprise a part of the present invention, and is therefore to be accorded such latitude and scope of interpretation.

As noted above, the functional activity of the OB polypeptide can be evaluated 10 transgenically. In this respect, a transgenic mouse model can be used. The ob gene can be used in complementation studies employing transgenic mice. Transgenic vectors, including viral vectors, or cosmid clones (or phage clones) corresponding to the wild type locus of candidate gene, can be constructed using the isolated ob gene. Cosmids may be introduced into transgenic mice using published procedures 15 [Jaenisch, Science, 240:1468-1474 (1988)]. The constructs are introduced into fertilized eggs derived from an intercross between F1 progeny of a C57BL/6J ob/ob X DBA intercross. These crosses require the use of C57BL/6J ob/ob ovarian transplants to generate the F1 animals. DBA/2J mice are used as the counterstrain because they have a nonagouti coat color which is important when using the ovarian transplants. Genotype at the ob loci in cosmid transgenic animals can be determined by typing animals with tightly linked RFLPs or microsatellites which flank the mutation and which are polymorphic between the progenitor strains. Complementation will be demonstrated when a particular construct renders a genetically obese F2 animal (as scored by RFLP analysis) lean and nondiabetic. Under these circumstances, final proof of complementation will require that the ob/ob or db/db animal carrying the transgene be mated to the ob/ob or db/db ovarian transplants. In this cross, all N2 animals which do not carry the transgene will be obese and insulin resistant/diabetic, while those that do carry the transgene will be lean and have normal glucose and insulin concentrations in plasma. In a genetic 30 sense, the transgene acts as a suppressor mutation.

Alternatively, OB genes can be tested by examining their phenotypic effects when expressed in antisense orientation in wild-type animals. In this approach, expression of the wild-type allele is suppressed, which leads to a mutant phenotype. RNARNA duplex formation (antisense-sense) prevents normal handling of mRNA, resulting in partial or complete elimination of wild-type gene effect. This technique has been used to inhibit TK synthesis in tissue culture and to produce phenotypes of the Kruppel mutation in Drosophila, and the Shiverer mutation in mice Izant et al., Cell, 36:1007-1015 (1984); Green et al., Annu. Rev. Biochem., 55:569-597 (1986); Katsuki et al., Science, 241:593-595 (1988). An important advantage of this approach is that only a small portion of the gene need be expressed for effective inhibition of expression of the entire cognate mRNA. The antisense transgene will be placed under control of its own promoter or another promoter expressed in the correct cell type, and placed upstream of the SV40 polyA site. This transgene will be used to make transgenic mice. Transgenic mice will also be mated ovarian transplants to test whether ob heterozygotes are more sensitive to the effects of the antisense construct.

In the long term, the elucidation of the biochemical function of the OB gene product (the OB polypeptide or protein) is useful for identifying small molecule agonists and antagonists that affect its activity.

Various terms used throughout this specification shall have the definitions set out 20 herein, for example, below.

The term "body weight modulator", "modulator", "modulators", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refers in one instance to both nucleotides and to proteinaceous material, the latter including both single or multiple proteins. More specifically, the aforementioned terms extend to the nucleotides and to the DNA having the sequences described herein and presented in Figure 1A through E (SEQ ID NO:1), and Figure 2A and B (SEQ ID NO:3). Likewise, the proteins having the

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amino acid sequence data described herein and presented in Figure 1A through E (SEQ ID NO:2), and Figure 3 (SEQ ID NO:4) are likewise contemplated, as are the profile of activities set forth with respect to all materials both herein and in the claims. Accordingly, nucleotides displaying substantially equivalent or altered activity are likewise contemplated, including substantially homologous analogs and allelic variations. Likewise, proteins displaying substantially equivalent or altered activity, including proteins modified deliberately, as for example, by site-directed mutagenesis, or accidentally through mutations in hosts that produce the modulators are likewise contemplated.

10 A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc., but excluding racemic forms of A) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

The OB Polypeptides

The terms "protein," which refers to the naturally occurring polypeptide, and "polypeptide" are used herein interchangeably with respect to the *ob* gene product and variants thereof. The term "mature protein" or "mature polypeptide" particularly refers to the *OB* gene product with the signal sequence (or a fusion protein partner) 25 removed.

As noted above, in specific embodiments ob polypeptides of the invention include those having the amino acid sequences set forth herein e.g., SEQ ID NOS: 2, 4, 5, 6, etc., including the ob polypeptide modified with conservative amino acid

substitutions, as well as biologically active fragments, analogs, and derivatives thereof. The term "biologically active," is used herein to refer to a specific effect of the polypeptide, including but not limited to specific binding, e.g., to a receptor, antibody, or other recognition molecule; activation of signal transduction pathways 5 on a molecular level; and/or induction (or inhibition by antagonists) of physiological effects mediated by the native ob polypeptide in vivo. OB polypeptides, including fragments, analogs, and derivatives, can be prepared synthetically, e.g., using the well known techniques of solid phase or solution phase peptide synthesis. Preferably. solid phase synthetic techniques are employed. Alternatively, OB polypeptides of the 10 invention can be prepared using well known genetic engineering techniques, as described infra. In yet another embodiment, the OB polypeptide can be purified, e.g., by immunoaffinity purification, from a biological fluid, such as but not limited to plasma, serum, or urine, preferably human plasma, serum, or urine, and more preferably from a subject who overexpresses the polypeptide, such as an obese person 15 suffering from a mutation in the OB receptor or from obesity related to a mutation corresponding to "fatty."

Fragments of the OB Polypeptide

In a particular embodiment, the present invention contemplates that naturally occurring fragments of the OB polypeptide may be important. The peptide sequence includes a number of sites that are frequently the target for proteolytic cleavage, e.g., arginine residues. It is possible that the full length polypeptide may be cleaved at one or more such sites to form biologically active fragments. Such biologically active fragments may either agonize or antagonize the functional activity of the OB polypeptide to reduce body weight.

Analogs of the OB Polypeptide

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The present invention specifically contemplates preparation of analogs of the OB peptide, which are characterized by being capable of a biological activity of OB polypeptide, e.g., of binding to a specific binding partner of ob peptide, such as the OB receptor. In one embodiment, the analog agonizes OB activity, i.e., it functions

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similarly to the ob peptide. Preferably, an OB agonist is more effective than the native protein. For example, an OB agonist analog may bind to the OB receptor with higher affinity, or demonstrate a longer half-life in wiw, or both. Nevertheless, OB peptide agonist analogs that are less effective than the native protein are also contemplated. In another embodiment, the analog antagonizes OB activity. For example, an OB analog that binds to the OB receptor but does not induce signal transduction can competitively inhibit binding of native OB to the receptor, thus decreasing OB activity in vivo. Such an OB antagonist analog may also demonstrate different properties from ob peptide, e.g., longer (or shorter) half-life in vivo, greater (or lesser) binding affinity for the OB receptor, or both.

In one embodiment, an analog of OB peptide is the OB peptide modified by substitution of amino acids at positions on the polypeptide that are not essential for structure or function. For example, since it is known that human OB peptide is biologically active in mouse, substitution of divergent amino acid residues in the human sequence as compared to the murine amino acid sequence will likely yield useful analogs of OB peptide. For example, the serine residue at position 53 or position 98, or both (in the unprocessed peptide sequence depicted in Figure 4) from human may be substituted, e.g., with glycine, alanine, valine, cysteine, methionine, or threonine. Similarly, the arginine residue at position number 92 (Figure 4) may 20 be substituted, e.g., with asparagine, lysine, histidine, glutamine, glutamic acid, aspartic acid, serine, threonine, methionine, or cysteine. Referring still to Figure 4. other amino acids in the human OB peptide that appear to be capable of substitution are histidine at position 118, tryptophan at position 121, alanine at position 122, glutamic acid at position 126, threonine at position 127, leucine at position 128, glycine at position 132, glycine at position 139, tryptophan at position 159, and glycine at position 166. In another embodiment, it may be possible to substitute one or more of residues 121 to 128 (as depicted in Figure 4), e.g., with glycines or alanines, or substituting some of the residues with the exceptions of serine as position 123, or leucine at position 125.

In another embodiment, an analog of the OB polypeptide, preferably the human OB polypeptide, is a truncated form of the polypeptide. For example, it has already been demonstrated that the glutamine at residue 49 is not essential, and can be deleted from the peptide. Similarly, it may be possible to delete some or all of the divergent 5 amino acid residues at positions 121-128. In addition, the invention contemplates providing an OB analog having the minimum amino acid sequence necessary for a biological activity. This can be readily determined, e.g., by testing the activity of fragments of OB for the ability to bind to OB-specific antibodies, inhibit the activity of the native OB polypeptide, or agonize the activity of the native OB peptide. In one 10 embodiment, the invention provides a truncated OB polypeptide consisting of the loop structure formed by the disulfide bond that forms between cysteine residues 117 and 167 (as depicted in Figure 4). In another embodiment, the truncated analog corresponds to the amino acids from residue 22 (which follows the putative signal peptide cleavage site) to 53 (the amino acid residue immediately preceding a flexible 15 loop region detected with limited proteolysis followed by mass spectrometric analysis of the OB polypeptide; see Cohen et al., Protein Science, 4:1088 (1995). In another embodiment, the truncated analog corresponds to amino acids from residue 61 (the residue immediately following the flexible loop region as detected with the limited proteolysis/mass spec. analysis of the OB polypeptide) to amino acid residue 116 (the 20 residue immediately preceding the first cysteine residue). In yet another embodiment, the truncated analog corresponds to amino acids from residue 61 to amino acid residue 167.

Furthermore, one or more of the residues of the putative flexible loop at residues number 54 to 60 are substituted. For example, one or more of the residues may be substituted with lysine, glutamic acid, or cysteine (preferably lysine) for cross linking, e.g., to a polymer, since flexible loop structures are preferred sites for derivatization of a protein. Alternatively, the residues at the flexible loop positions may be substituted with amino acid residues that are more resistant to proteolysis but that retain a flexible structure, e.g., one or more prolines. In yet another embodiment,

substitutions with amino acid residues that can be further derivatized to make them more resistant to degradation, e.g., proteolysis, is contemplated.

It will be appreciated by one of ordinary skill in the art that the foregoing fragment sizes are approximate, and that from one to about five amino acids can be included 5 or deleted from each or both ends, or from the interior of the polypeptide or fragments thereof, of the recited truncated analogs, with the exception that in the disulfide bonded loop analogs, the cysteine residues must be maintained.

It has been found that murine OB peptide contains 50% α-helical content, and that the human OB polypeptide contains about 60% α-helical content, as detected by 10 circular dichroism of the recombinant peptides under nearly physiological conditions. Accordingly, in another embodiment, amino acid residues can be substituted with residues to form analogs of OB polypeptide that demonstrate enhanced propensity for forming, or which form more stable, α -helix structures. For example, α -helix structure would be preferred if Glu, Ala, Leu, His, Trp are introduced as substitutes 15 for amino acid residues found in the native OB polypeptide. Preferably, conservative amino acid substitutions are employed, e.g., substituting aspartic acid at residue(s) 29, 30, 44, 61, 76, 100, and/or 106 (as depicted in Figure 4) with glutamic acid(s) (Glu); substituting isoleucine(s) with leucine; substituting glycine or valine, or any divergent amino acid, with alanine (e.g., serine at position 53 of the human OB 20 polypeptide with alanine), substituting arginine or lysine with histidine, and substituting tyrosine and/or phenylalanine with tryptophan. Increasing the degree, or more importantly, the stability of α -helix structure may yield an OB analog with greater activity, increased binding affinity, or longer half-life. In a specific embodiment, the helix forming potential of the portion of the OB peptide 25 corresponding to amino acid residues 22 through 53 is increased. In another embodiment, the helix-forming potential or stability of the amino acid residues 61-116 is increased. In yet another embodiment, the helix forming potential of the disulfide loop structure corresponding to amino acids 117 to 167 is increased. Also contemplated are OB analogs containing enhanced \alpha-helical potential or stability in

more than one of the foregoing domains. In a further embodiment, truncated OB polypeptide analogs are generated that incorporate structure-forming, e.g., helix-forming, amino acid residues to compensate for the greater propensity of polypeptide fragments to lack stable structure.

5 Analogs, such as fragments, may be produced, for example, by pepsin digestion of weight modulator peptide material. Other analogs, such as muteins, can be produced by standard site-directed mutagenesis of weight modulator peptide coding sequences. Analogs exhibiting "weight modulator activity" such as small molecules, whether functioning as promoters or inhibitors, may be identified by known in vivo and/or in viro assays.

Small Molecule Analogs and Peptidomimetics of OB Polypeptide

The structure of the ob polypeptide, preferably human OB polypeptide, can be analyzed by various methods known in the art. The protein sequence can be characterized by a hydrophilicity analysis [e.g., Hopp et al., Proc. Natl. Acad. Sci.

15 USA, 78:3824 (1981)]. A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the OB polypeptide, which may indicate regions buried in the interior of the folded polypeptide, and regions accessible on the exterior of the polypeptide. In addition, secondary structural analysis [e.g., Chou et al., Biochem., 13:222 (1974)] can also be done, to identify regions of OB polypeptide that assume specific secondary structures. Manipulation of the predicted or determined structure, including secondary structure prediction, can be accomplished using computer software programs available in the art.

By providing an abundant source of recombinant OB polypeptide, the present invention enables quantitative structural determination of the polypeptide. In particular, enough material is provided for nuclear magnetic resonance (NMR), infrared (IR), Raman, and ultraviolet (UV), especially circular dichroism (CD), spectroscopic analysis. In particular NMR provides very powerful structural analysis of molecules in solution, which more closely approximates their native environment

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[Marion et al., Biochim. Biophys. Res. Comm., 113:967-974 (1983); Bar et al., J. Magn. Reson., 65:355-360 (1985); Kimura et al., Proc. Natl. Acad. Sci. USA, 77:1681-1685 (1980)]. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography [Engstom, Biochem Exp. 5 Biol., 11:7-13 (1974)].

In yet a further embodiment, an analog of OB polypeptide can be tested to determine whether it cross-reacts with an antibody specific for native OB polypeptide, or specific fragments thereof. The degree of cross-reactivity provides information about structural homology or similarity of proteins, or about the accessibility of regions corresponding to portions of the polypeptide that were used to generate fragment-specific antibodies.

Screening for OB Analogs

Various screening techniques are known in the art for screening for analogs of polypeptides. Various libraries of chemicals are available. Accordingly, the present invention contemplates screening such libraries, e.g., libraries of synthetic compounds generated over years of research, libraries of natural compounds, and combinatorial libraries, as described in greater detail, infra, for analogs of OB polypeptide. In one embodiment, the invention contemplates screening such libraries for compounds that bind to anti-OB polypeptide antibodies, preferably anti-human ob polypeptide antibodies. In another aspect, once the OB receptor is identified (see infra), any screening technique known in the art can be used to screen for OB receptor agonists or antagonists. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and agonize or antagonize activate OB receptor in vivo.

25 Knowledge of the primary sequence of the receptor, and the similarity of that sequence with proteins of known function, can provide an initial clue as to the agonists or antagonists of die protein. Identification and screening of antagonists is further facilitated by determining structural features of the protein, e.g., using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and

other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" [Scott et al., Science, 249:386-390 (1990); Cwirla et al., Proc. 5 Natl. Acad. Sci. USA, 87:6378-6382 (1990); Devlin et al., Science, 249:404-406 (1990)], very large libraries can be constructed (106-104 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method [Geysen et al., Molecular Immunology, 23:709-715 (1986); Geysen et al., J. Immunologic Method, 102:259-274 (1987)] and the recent method of Fodor et al.. Science. 10 251:767-773 (1991) are examples. Furka et al. 14th International Congress of

Biochemistry, Volume 5, Abstract FR:013 (1988); Furka, Int. J. Peptide Protein Res., 37:487-493 (1991)]; Houghton (U.S. Patent No. 4,631,211, issued December 1986); and Rutter et al. (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

15 In another aspect, synthetic libraries [Needels et al., Proc. Natl. Acad. Sci. USA, 90:10700-10704 (1993); Lam et al., International Patent Publication No. WO 92/00252, each of which is incorporated herein by reference in its entirety], and the like can be used to screen for OB receptor ligands according to the present invention. With such libraries, receptor antagonists can be detected using cells that express the 20 receptor without actually cloning the OB receptor.

Alternatively, assays for binding of soluble ligand to cells that express recombinant forms of the OB receptor ligand binding domain can be performed. The soluble ligands can be provided readily as recombinant or synthetic OB polypeptide.

The screening can be performed with recombinant cells that express the OB receptor, 25 or alternatively, using purified receptor protein, e.g., produced recombinantly, as described above. For example, the ability of labeled, soluble or solubilized OB receptor, that includes the ligand-binding portion of the molecule, to bind ligand can be used to screen libraries, as described in the foregoing references.

Derivatives of OB Polypeptides

Generally, the present protein (herein the term "protein" is used to include

5 "polypeptide," unless otherwise indicated) may be derivatized by the attachment of
one or more chemical moieties to the protein moiety. The chemically modified
derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular,
subcutaneous, intravenous, oral, nasal, rectal, bucal, sublingual, pulmonary, topical,
transdermal, or other routes of administration. Chemical modification of biologically
active proteins has been found to provide additional advantages under certain
circumstances, such as increasing the stability and circulation time of the therapeutic
protein and decreasing immunogenicity. See U.S. Patent No. 4,179,337, Davis et
al., issued December 18, 1979. For a review, see Abuchowski et al., "Soluble
Polymer-Enzyme Adducts", in Enzymes as Drugs, pp. 367-383, Holcenberg and
15 Roberts, eds., Wiley-Interscience, New York, NY, (1981). A review article
describing protein modification and fusion proteins is Francis, Focus on Growth
Factors, 3:4-10 (1992).

Chemical Moieties For Derivatization

The chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present proteins and peptides, these may be ascertained using the assays provided herein.

Polymer Molecules

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldenhyde may provide advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

Polymer/Protein Ratio

20 The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

Attachment of the Chemical Moiety to the Protein

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the 5 art, e.g., EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF). See also Malik et al., Exp. Hematol., 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene givcol molecule may be bound. The amino acid residues having a free amino group may 10 include lysine residues and the N-terminal amino acid residues, those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydry groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or 15 lysine group. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

N-terminally Chemically Modified Proteins.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino

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groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pK, differences between the ϵ -amino groups of the lysine residues and that of the α -amino group of the N-terminal residue of the protein. By such selective derivatization attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

Nucleic Acids Associated With OB Polypeptide

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As noted above, the present invention is directed to nucleic acids encoding ob polypeptides, as well as associated genomic non-coding sequences 5', 3', and intronic to the OB gene. Thus, in accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., DNA Cloning: A Practical Approach, Volumes I and II, MRL Press, Ltd., Oxford. U.K. (1985); Gait ed., Oligonucleotide Synthesis, Oxford University Press (1984); Hames et al., eds., Nucleic Acid Hybridization, Springer-Verlag (1985); Hames et al., eds. Transcription And Translation, Oxford University Press (1984)]; Freshney ed., Animal Cell Culture. Oxford University Press (1986)]; Immobilized Cells And Enzymes, IRL Press (1986)]; Perbal, A Practical Guide To Molecular Cloning, Wiley, New York (1984). Of particular relevance to the present invention are strategies for isolating, cloning, sequencing, analyzing, and

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characterizing a gene or nucleic acid based on the well known polymerase chain reaction (PCR) techniques.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo, i.e., capable of replication under its own control

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

- 15 A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.
- 20 A "clone" is a population of cells derived from a single cell or common ancestor by mitosis.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single-stranded form, or a double-stranded helix. Double-stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary or quaternary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

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A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook et al., 1989, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°C, can be used, e.g., 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m , e.g., 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, e.g., 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:RNA,

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

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Isolation of OB Coding and Flanking Sequences

The nucleic acids contemplated by the present invention extend as indicated, to other nucleic acids that code on expression for peptides such as those set forth in FIGURE 1A through D (SEQ ID NO:2), FIGURE 3 (SEQ ID NO:4), FIGURE 5 (SEQ ID NO:5), and FIGURE 6 (SEQ ID NO:6) herein. Accordingly, while specific DNA has been isolated and sequenced in relation to the ob gene, any animal cell potentially can serve as the nucleic acid source for the molecular cloning of a gene encoding the peptides of the invention. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook et al., 1989, supra; Glover, 1985, supra). Clones derived from genomic DNA may contain regulatory and intronic DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, the genomic DNA can be amplified using primers selected from the cDNA sequences. Alternatively, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. One may also use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired ob or ob-like gene may be accomplished in a number of ways. For example, if an amount of a portion of a ob or ob-like gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to a labeled probe [Benton et al., Science, 196:180 (1977); Grunstein et al., Proc. Natl. Acad. Sci.

USA, 72:3961 (1975)]. The present invention provides such nucleic acid probes. which can be conveniently prepared from the specific sequences disclosed herein, e.g., a hybridizable probe having a nucleotide sequence corresponding to at least a 10, and preferably a 15, nucleotide fragment of the sequences depicted in Figure 1A 5 through E (SEO ID NO:1) or Figure 2A and B (SEO ID NO:3). Preferably, a fragment is selected that is highly unique to the modulator peptides of the invention. Those DNA fragments with substantial homology to the probe will hybridize. As noted above, the greater the degree of homology, the more stringent the hybridization conditions that can be used. In one embodiment, low stringency hybridization 10 conditions are used to identify a homologous modulator peptide. However, in a preferred aspect, and as demonstrated experimentally herein, a nucleic acid encoding a modulator peptide of the invention will hybridize to a nucleic acid having a nucleotide sequence such as depicted in Figure 1A through E (SEQ ID NO:1) or Figure 2A and B (SEO ID NO:3), or a hybridizable fragment thereof, under 15 moderately stringent conditions; more preferably, it will hybridize under high stringency conditions.

Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, e.g., has similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, tyrosine phosphatase activity or antigenic properties as known for the present modulator peptides. For example, the antibodies of the instant invention can conveniently be used to screen for homologs of modulator peptides from other sources.

25 A gene encoding a modulator peptide of the invention can also be identified by mRNA selection, i.e., by nucleic acid hybridization followed by in vitro translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified modulator DNA. Immunoprecipitation analysis or functional assays (e.g., tyrosine phosphatase)

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activity) of the *in vitro* translation products of the products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments, that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against a modulator peptide.

A radiolabeled modulator peptide cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify homologous modulator peptide DNA fragments from among other genomic DNA fragments.

10 As mentioned above, a DNA sequence encoding weight modulator peptides as disclosed herein can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the weight modulator peptide amino acid sequences. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, Nature, 292:756 (1981); Nambair et al., Science, 223:1299 (1984); Jay et al., J. Biol. Chem., 259:6311 (1984).

Synthetic DNA sequences allow convenient construction of genes which will express weight modulator analogs, as described above. Alternatively, DNA encoding analogs can be made by site-directed mutagenesis of native OB genes or cDNAs, and analogs can be made directly using conventional polypeptide synthesis.

A general method for site-specific incorporation of unnatural amino acids into proteins is described in Noren et al. Science, 244:182-188 (1989). This method may be used to create analogs of the ob polypeptide with unnatural amino acids.

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Non-coding Nucleic Acids

The present invention extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of the weight modulator proteins at the translational level. This approach utilizes antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule [See Weintraub, Sci. Am., 262:40-46 (1990); Marcus-Sekura, Anal. Biochem., 172:289-295 (1988)]. In the cell, they hybridize to that mRNA, forming a double-stranded molecule. The cell does not translate an mRNA complexed in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into weight modulator peptide-producing cells. Antisense methods have been used to inhibit the expression of many genes in vitro [(Marcus-Sekura, 1988 supra; Hambor et al., J. Exp. Med., 168:1237-1245 (1988)].

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it [Cech, J. Am. Med. Assoc., 260:3030-3034 (1988)]. Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

Investigators have identified two types of ribozymes, Tetrahymena-type and "hammerhead"-type. Tetrahymena-type ribozymes recognize four-base sequences,

while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target Therefore, hammerhead-type ribozymes are preferable to mRNA species. Tetrahymena-type ribozymes for inactivating a specific mRNA species, and eighteen 5 base recognition sequences are preferable to shorter recognition sequences.

The DNA sequences described herein may thus be used to prepare antisense molecules against and ribozymes that cleave mRNAs for weight modulator proteins and their ligands, thus inhibiting expression of the ob gene, and leading to increased weight gain and adiposity.

10 In another embodiment, short oligonucleotides complementary to the coding and complementary strands of the OB nucleic acid, or to non-coding regions of the OB gene 5', 3', or internal (intronic) to the coding region are provided by the present invention. Such nucleic acids are useful as probes, either as directly labeled oligonucleotide probes, or as primers for the polymerase chain reaction, for 15 evaluating the presence of mutations in the ob gene, or the level of expression of OB mRNA. Preferably, the non-coding nucleic acids of the invention are from the human OB gene.

In a specific embodiment, the non-coding nucleic acids provide for homologous recombination for integration of an amplifiable gene and/or other regulatory 20 sequences in proximity to the OB gene, e.g., to provide for higher levels of expression of the OB polypeptide, or to overcome a mutation in the ob gene regulatory sequences that prevent proper levels of expression of the OB polypeptide (See International Patent Publication WO 91/06666, published May 16, 1991 by Skoultchi; International Patent Publication No. WO 91/09955, published July 11, 25 1991 by Chappel; see also International Patent Publication No. WO 90/14092,

published November 29, 1990, by Kucherlapati and Campbell).

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Production of OB Polypeptide: Expression and Synthesis

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation 5 signals are control sequences.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

10 A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is also used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted upstream (5') of and in reading frame with the gene.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding

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sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease \$1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular bott

Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., E. coli plasmids col El, pCR1, pBR322, pMB9, pUC or pUC plasmid derivatives, e.g., pGEX vectors, pET vectors, pmal-c, pFLAG, etc., and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single-stranded phage DNA; yeast plasmids such as the 2μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences;

and the like. In a preferred embodiment, expression of ob is achieved in methylotrophic yeast, e.g., Pichia pastoris yeast (see, e.g., International Patent Publication No. WO 90/03431, published 5 April 1990, by Brierley et al.; International Patent Publication No. WO 90/10697, published 20 September 1990, by
5 Siegel et al.). In a specific embodiment, infra, an expression vector is engineered for expression of ob under control of the α-matting factor signal sequence.

Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the AOX l promoter of methylotrophic yeast, the promoters of the yeast α-mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and 20 prokaryotic hosts, such as strains of *E. coli, Pseudomonas, Bacillus, Streptomyces*; fungi such as yeasts (*Saccharomyces*, and methylotrophic yeast such as *Pichia*, *Candida, Hansenula*, and *Torulopsis*); and animal cells, such as CHO, Rl.I, B-W and LM cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue

It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one

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skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

Considering these and other factors, a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

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In a specific embodiment, an OB fusion protein can be expressed. An OB fusion protein comprises at least a functionally active portion of a non-OB protein joined via a peptide bond to at least a functionally active portion of an OB polypeptide. The non-ob sequences can be amino- or carboxy-terminal to the OB sequences. More preferably, for stable expression of a proteolytically inactive OB fusion protein, the portion of the non-OB fusion protein is joined via a peptide bond to the amino-terminus of the OB protein. A recombinant DNA molecule encoding such a fusion protein comprises a sequence encoding at least a functionally active portion of a non-

OB protein joined in-frame to the OB coding sequence, and preferably encodes a cleavage site for a specific protease, e.g., thrombin or Factor Xa, preferably at the OB-non-OB juncture. In a specific embodiment, the fusion protein is expressed in Escherichia coli or in P. pastoris.

5 In a specific embodiment, infra, vectors were prepared to express the murine and human ob genes, with and without the codon for gln-49, in bacterial expression systems and yeast (Pichia) expression systems as fusion proteins. The ob gene is prepared with an endonuclease cleavage site, e.g., using PCR and novel primers. It is desirable to confirm sequences generated by PCR, since the probability of including a point mutation is greater with this technique. A plasmid containing a histidine tag (His-tag) and a proteolytic cleavage site is used. The presence of the histidine makes possible the selective isolation of recombinant proteins on a Ni-chelation column, or by affinity purification. The proteolytic cleavage site, in a specific embodiment, infra, a thrombin cleavage site, is engineered so that treatment with the protease, e.g., thrombin, will release the full-length mature (i.e., lacking a signal sequence) OB polypeptide.

In another aspect, the pGEX vector [Smith et al., Gene 67:31-40 (1988)] can be used. This vector fuses the schistosoma japonicum glutathionine S-transferase cDNA to the sequence of interest. Bacterial proteins are harvested and recombinant proteins can be quickly purified on a reduced glutathione affinity column. The GST carrier can subsequently be cleaved from fusion proteins by digestion with site-specific proteases. After cleavage, the carrier and uncleaved fusion protein can be removed by absorption on glutathione agarose. Difficulty with the system occasionally arises when the encoded protein is insoluble in aqueous solutions.

Expression of recombinant proteins in bacterial systems may result in incorrect folding of the expressed protein, requiring refolding. The recombinant protein can be refolded prior to or after cleavage to form a functionally active OB polypeptide. The OB polypeptide may be refolded by the steps of (i) incubating the protein in a

denaturing buffer that contains a reducing agent, and then (ii) incubating the protein in a buffer that contains an oxidizing agent, and preferably also contains a protein stabilizing agent or a chaotropic agent, or both. Suitable redox (reducing/oxidizing) agent pairs include, but are not limited to, reduced glutathione/glutathione disulfide. 5 cystine/cysteine, cystamine/cysteamine, and 2-mercaptoethanol/2hydroxyethyldisulfide. In a particular aspect, the fusion protein can be solubilized in a denaturant, such as urea, prior to exchange into the reducing buffer. In preferred embodiment, the protein is also purified, e.g., by ion exchange or Ni-chelation chromatography, prior to exchange into the reducing buffer. Denaturing agents 10 include but are not limited to urea and guanidine-HCl. The recombinant protein is then diluted about at least 10-fold, more preferably about 100-fold, into an oxidizing buffer that contains an oxidizing agent, such as but not limited to 0.1 M Tris-HCl, pH 8.0. 1 mM EDTA, 0.15 M NaCl, 0.3 M oxidized glutathione. The fusion protein is then incubated for about 1 to about 24 hours, preferably about 2 to about 16 hours, 15 at room temperature in the oxidizing buffer. The oxidizing buffer may comprise a protein stabilizing agent, e.g., a sugar, an alcohol, or ammonium sulfate. The oxidizing buffer may further comprises a chaotropic agent at low concentration, to destabilize incorrect intermolecular interactions and thus promote proper folding. Suitable chaotropic agents include but are not limited to a detergent, a polyol, L-20 arginine, guanidine-HCl and polyethylene glycol (PEG). It is important to use a low enough concentration of the chaotropic agent to avoid denaturing the protein. The refolded protein can be concentrated by at least about 10-fold, more preferably by the amount it was diluted into the oxidizing buffer.

Bacterial fermentation processes can also result in a recombinant protein preparation
that contains unacceptable levels of endotoxins. Therefore, the invention
contemplates removal of such endotoxins, e.g., by using endotoxin-specific antibodies
or other endotoxin binding molecules. The presence of endotoxins can be determined
by standard techniques, such as by employing E-TOXATE Reagents (Sigma, St.
Louis, Missouri), or with bioassays.

In addition to the specific example, the present inventors contemplate use of baculovirus, mammalian, and yeast expression systems to express the ob protein. For example, in baculovirus expression systems, both non-fusion transfer vectors, such as but not limited to pVL941 (BamH1 cloning site; Summers), pVL1393 (BamH1, 5 SmaI, XbaI, EcoR1, Not1, XmaIII, Bg/II, and PsrI cloning site; Invitrogen), pVL1392 (Bg/II, PsrI, Not1, XmaIII, EcoR1, XbaI, SmaI, and BamH1 cloning site; Summers and Invitrogen), and pBlueBacIII (BamH1, Bg/II, PsrI, NcoI, and HindIII cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (BamH1 and KprI cloning site, in which the BamH1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (BamH1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with BamH1, Bg/II, PsrI, NcoI, and HindIII cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)).

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, e.g., any expression vector with a DHFR expression vector, or a DHFR/methotrexate coamplification vector, such as pED (PstI, SalI, SbaI, SmaI, and EcoRI cloning site, with the vector expressing both the cloned gene and DHFR; see Kaufman, Current 20 Protocols in Molecular Biology, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (HindIII, XbaI. SmaI. SbaI. EcoRI. and BcII cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (BamH1, Sfil, Xhol, Norl, Nhel, HindIII, Nhel, PvuII, and KpnI cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (BamH1, Sfil, Xhol, Notl, Nhel, HindIII, Nhel, PvuII, and KpnI cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (KpnI, PvuI, NheI, HindIII, NotI, XhoI, SfiI, BamH1 cloning

site. inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (BamH1, Xhol, Norl, HindIII, Nhel, and Kpnl cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (Kpnl, Nhel, HindIII, Norl, Xhol, Sfil, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (HindIII, BstXI, Norl, Sbal, and Apal cloning site, G418 selection; Invitrogen), pRc/RSV (HindIII, Spel, BstXI, Norl, Xbal cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (see, Kaufman, 1991, supra) for use according to the invention include but are not limited to pSC11 (Smal cloning site, TK- and β-gal selection), pMJ601 (Sall, Smal, AfII, Narl, BspMII, BamHI, Apal, Nhel, SacII, Kpnl, and HindIII cloning site; TK- and β-gal selection), and pTKgptF1S (EcoRl, Pstl, Sall, Accl, HindII, Sbal, BamHI, and 15 Hpa cloning site. TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express OB polypeptide. For example, the non-fusion pYES2 vector (XbaI, SphI, ShoI, NotI, GstXI, EcoRI, BstXI, BamH1, SacI, Kpn1, and HindIII cloning sit; Invitrogen) or the fusion pYESHisA, B, C (XbaI, SphI, ShoI, NotI, BstXI, EcoRI, BamH1, SacI, KpnI, and HindIII cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

It is further intended that body weight modulator peptide analogs may be prepared from nucleotide sequences derived within the scope of the present invention.

25 In addition to recombinant expression of OB polypeptide, the present invention envisions and fully enables preparation of OB polypeptide, or fragments thereof, using the well known and highly developed techniques of solid phase peptide synthesis. The invention contemplates using both the popular Boc and Fmoc, as well

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as other protecting group strategies, for preparing ob polypeptide or fragments thereof. Various techniques for refolding and oxidizing the cysteine side chains to form a disulfide bond are also well-known in the art.

Antibodies to the OB Polypeptide

- 5 According to the invention, OB polypeptide produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the OB polypeptide. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.
- 10 A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, i.e., capable of eliciting an immune response without a carrier.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567, as well as antigen binding portions of antibodies, including Fab, F(ab'), and F(v) (including single chain antibodies). Accordingly, the phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule containing the antibody combining site. An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v), which portions are preferred for use in the therapeutic methods described herein.

Fab and F(ab')₂ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response [Hood et al., in Immunology, p. 384, Second Ed., Penjamin/Cummings, Menlo Park, California (1984)]. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as

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aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Preferably, the adjuvant is pharmaceutically acceptable.

Various procedures known in the art may be used for the production of polyclonal antibodies to OB polypeptide, or fragment, derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the OB polypeptide, or a derivative (e.g., fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the OB polypeptide or fragment thereof can be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of monoclonal antibodies directed toward the OB polypeptide, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler et al., Nature, 256:495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., Immunology Today, 4:72 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in Monoclonal Antibodies and Cancer Therapy, pp. 77-96. Alan R. Liss, Inc., (1985)]. Immortal, antibody-producing cell lines can be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., "Hybridoma

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Techniques" (1980); Hammerling et al., "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890.

- 5 In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas [Cote et al., Proc. Natl. Acad. Sci. USA, 80:2026-2030 (1983)] or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, supra). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et al., J. Bacteriol., 159-870 (1984); Neuberger et al., Nature, 312:604-608 (1984); Takeda et al., Nature, 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for an ob polypeptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described infra), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.
- 20 According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce OB polypeptide-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., Science, 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for an ob polypeptide, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab'), fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab'), fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5 In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g., radioimmunoassay, ELISA "sandwich" (enzyme-linked immunosorbent assay), immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for 10 example), Western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting 15 binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of an OB polypeptide, one may assay generated hybridomas for a product which binds to 20 an OB polypeptide fragment containing such epitope. For selection of an antibody specific to an OB polypeptide from a particular species of animal, one can select on the basis of positive binding with OB polypeptide expressed by or isolated from cells of that species of animal.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the OB polypeptide, e.g., for Western blotting, imaging OB polypeptide in situ, measuring levels thereof in appropriate physiological samples, etc.

In a specific embodiment, antibodies that agonize or antagonize the activity of OB polypeptide can be generated. Such antibodies can be tested using the assays described *infra* for identifying ligands.

In a specific embodiment, antibodies are developed by immunizing rabbits with synthetic peptides predicted by the protein sequence or with recombinant proteins made using bacterial expression vectors. The choice of synthetic peptides is made after careful analysis of the predicted protein structure, as described above. In particular, peptide sequences between putative cleavage sites are chosen. Synthetic peptides are conjugated to a carrier such as KLH hemocyanin or BSA using carbodiimide and used in Freunds adjuvant to immunize rabbits. In order to prepare recombinant protein, the pGEX vector can be used to express the polypeptide (Smith et al., 1988, supra). Alternatively, one can use only hydrophilic domains to generate the fusion protein. The expressed protein will be prepared in quantity and used to immunize rabbits in Freunds adjuvant.

15 In another specific embodiment, recombinant OB polypeptide is used to immunize chickens, and the chicken anti-OB antibodies are recovered from egg yolk, e.g., by affinity purification on an OB-column. Preferably, chickens used in immunization are kept under specific pathogen free (SPF) conditions.

In another embodiment, antibodies against leptin are generated in ob/ob mice, which lack circulating OB protein, and thus are expected to be capable of generating an anti-OB polypeptide response since they will not be tolerized to the polypeptide, and wild-type mice. Spleen cells from both groups of mice can be fused with myeloma cells to prepare hybridomas for monoclonal antibodies.

In yet another embodiment, recombinant OB polypeptide is used to immunize rabbits.

25 and the polyclonal antibodies are immunopurified prior to further use. The purified antibodies are particularly useful for semi-quantitative assays, particularly for detecting the presence of circulating OB polypeptide in serum or plasma.

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Panels of monoclonal antibodies produced against modulator peptides can be screened for various properties; i.e., isotype, epitope, affinity, etc. Of particular interest are monoclonal antibodies that neutralize the activity of the modulator peptides. Such monoclonals can be readily identified in activity assays for the weight modulators. High affinity antibodies are also useful when immunoaffinity purification of native or recombinant modulator is possible.

Preferably, the anti-modulator antibody used in the diagnostic and therapeutic methods of this invention is an affinity-purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-modulator antibody molecules used herein be in the form of Fab, Fab', F(ab'), or F(y) portions of whole antibody molecules.

Diagnostic Implications

The present invention also relates to a variety of diagnostic applications, including methods for detecting the presence of conditions and/or stimuli that impact upon abnormalities in body weight or adiposity, by reference to their ability to elicit the activities which are mediated by the present weight modulators. As mentioned earlier, the weight modulator peptides can be used to produce antibodies to themselves by a variety of known techniques, and such antibodies could then be isolated and utilized as in tests for the presence of particular transcriptional activity in suspect target cells. alternatively, the nucleic acids of the invention can be employed in diagnosis.

Antibody-based Diagnostics

As suggested earlier, a diagnostic method useful in the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to a modulator protein, such as an anti-modulator antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the anti-modulator antibody molecules used herein be in the form of Fab, Fab', F(ab'), or F(v) portions or whole antibody molecules. As

previously discussed, patients capable of benefiting from this method include those suffering from cancer, AIDS, obesity or other conditions where abnormal body weight is a characteristic or factor. Methods for isolating the modulator and inducing anti-modulator antibodies and for determining and optimizing the ability of anti-modulator antibodies to assist in the examination of the target cells are all well-known in the art.

Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of the weight control modulators and other recognition factors and/or their subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions where abnormalities in body weight are or may be likely to develop. For example, the modulator peptides or their active fragments may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques, such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. These techniques are described in detail below. Likewise, small molecules that mimic or antagonize the activity(ies) of the receptor recognition factors of the invention may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

The presence of weight modulators in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Three such procedures which are especially useful utilize either the receptor recognition factor labeled with a detectable label, antibody Ab, labeled with a detectable label, or antibody Ab, labeled with a detectable label. The procedures may be summarized by the following equations wherein the asterisk indicates that the particle is labeled, and "WM" stands for the weight modulator:

A.
$$WM^* + Ab_1 = WM^*Ab_1$$

$$B. WM + Ab_1 = WMAb_1*$$

C.
$$WM + Ab_1 + Ab_2 = Ab_1WMAb_2$$

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. Procedure B is representative of well known competitive assay techniques. Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody", or "DASP" procedure.

In each instance, the weight modulators form complexes with one or more antibody(ies) or binding partners and one member of the complex is labeled with a 10 detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels.

It will be seen from the above, that a characteristic property of Ab₂ is that it will react with Ab₁. This is because Ab₁, raised in one mammalian species, has been used in another species as an antigen to raise the antibody, Ab₂. For example, Ab₂ may be raised in goats using rabbit antibodies as antigens. Ab₂ therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims, Ab₁ will be referred to as a primary or anti-weight modulator antibody, and Ab₂ will be referred to as a secondary or anti-Ab, antibody.

The labels most commonly employed for these studies are radioactive elements, 20 enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others.

A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine and auramine. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

25 The weight modulators or their binding partners can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the WA 95/05309

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currently available counting procedures. The preferred isotope may be selected from ³H. ¹⁴C. ³²P. ³⁵S. ³⁶Cl. ³¹Cr. ⁵⁷Co. ³⁶Co. ³⁶Fe. ⁹⁰Y. ¹²⁵I, ¹³¹I, and ¹⁸⁶Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, \(\beta\)-D-glucosidase, \(\beta\)-D-glucosi

In a further embodiment of this invention, test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of predetermined transcriptional activity or predetermined transcriptional activity capability in suspected target cells. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labeled weight modulator or its binding partner, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

- 20 Accordingly, a test kit may be prepared for the demonstration of the presence or capability of cells for predetermined transcriptional activity, comprising:
 - (a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of the present weight modulator or a specific binding partner thereto, to a detectable label;
- 25 (b) other reagents; and
 - (c) directions for use of said kit.

More specifically, the diagnostic test kit may comprise:

- (a) a known amount of the weight modulator as described above (or a binding partner) generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each:
 - (b) if necessary, other reagents; and
 - (c) directions for use of said test kit.

In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. "competitive," 10 "sandwich." "double antibody." etc.), and comprises:

- (a) a labeled component which has been obtained by coupling the weight modulator to a detectable label:
- (b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group 15 consisting of:
 - (i) a ligand capable of binding with the labeled component (a);
 - (ii) a ligand capable of binding with a binding partner of the labeled component (a);
- (iii) a ligand capable of binding with at least one of the component(s) to 20 be determined: and
 - (iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and
- (c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between 25 the weight modulator and a specific binding partner thereto.

Nucleic Acid-based Diagnostics

As demonstrated in the examples, *infra*, nucleic acids of the invention can be used to detect defects associated with defects in the OB polypeptide that result in obese phenotypes. For example, nucleic acid probes (e.g., in Northern analysis or RT-PCR

analysis) can be used to determine whether an obese phenotype is associated with lack of expression of OB mRNA, or expression of non-functional OB mRNA, e.g., as in db/db mice (where the deficiency results from lack of an OB receptor) or where a mutation yields a non-transcribed mRNA. Moreover, the nucleic acid-based diagnostic techniques of the invention can be used in conjunction with antibody-based techniques to further develop a molecular understanding of obese or anorexic phenotypes.

The human cDNA clones that have recently been isolated have been sequenced as presented herein. This facilitates the determination of the complete sequence of the human gene (see Figure 20A through C; SEQ ID NO: 22). DNA sequences from the introns of the human OB gene have been obtained (Figure 20), and these have been used to prepare PCR primers to PCR amplify the coding sequence of the OB gene from human genomic DNA so as to identify mutations or allelic variants of the OB gene, all in accordance with protocols described in detail earlier herein. Specific PCR primers for amplifying human genomic OB are described in a specific Example, infra.

The current hypothesis is that heterozygous mutations in the ob gene will be associated with mild/moderate obesity while homozygous mutations would be associated with several DNA sequence-based diagnostic tests for obesity. If this is true, it would allow the ascertainment of people at risk for the development of obesity and make possible the application of drug treatment and/or lifestyle changes before an increased body weight is fully developed.

Alternatively, the presence of microsatellites that segregate with mutant forms of human OB can be used for diagnosis. Various PCR primers, including those based on the nucleotide sequence provided in Figure 20A through C, can be used in this respect.

The OB gene may also be useful diagnostically for measurements of its encoded RNA and protein in nutritional disorders. It will be of importance to know, in a particular nutritional disorder, whether OB RNA and/or its encoded protein is unregulated or downregulated. Thus, if an obese person has increased levels of OB, it would appear that the problem is downstream of OB, while if OB is reduced, it would appear that inappropriately low levels of OB may be cause of obesity (whether or not the defect is in the OB gene). Conversely, if a cancer or AIDS patient who lost weight had elevated levels of OB, it may be concluded that inappropriately high expression of OB is responsible for the weight loss.

10 The cloned human cDNA will be of use for the measurement of the levels of human OB RNA. In addition, recombinant human protein will be prepared and used to develop immunoassays to enable measurement of the fat and perhaps plasma levels of the OB protein.

Therapeutic Implications

15 The polypeptides, nucleic acids, and antibodies of the invention have significant therapeutic potential. Preferably, a therapeutically effective amount of such an agent is administered in a pharmaceutically acceptable carrier, diluent, or excipient.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or

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solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA. (1990).

5 The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant 0 condition in the host.

Administration of recombinant OB polypeptide results in weight loss, in particular, a decrease in fat tissue. OB polypeptide can be prepared using standard bacterial and/or mammalian expression vectors, synthetically, or purified from plasma or serum, all as stated in detail earlier herein. Alternatively, increased expression of native OB polypeptide may be induce by homologous recombination techniques, as described surra.

Reduction of OB polypeptide activity (by developing antagonists, inhibitors, use of neutralizing antibodies, or antisense molecules) should result in weight gain as might be desirable for the treatment of the weight loss associated with cancer, AIDS or anorexia nervosa. Modulation of OB activity can be useful for reducing body weight (by increasing its activity) or increasing body weight (by decreasing its activity).

Polypeptide-based Therapeutic Treatment

In the simplest analysis, the OB gene determines body weight in mammals, in particular, mice and man. The OB gene product, and, correspondingly, cognate molecules, appear to be part of a signaling pathway by which adipose tissue communicates with the brain and the other organs. It is believed that the OB polypeptide is itself a signaling molecule, i.e., a hormone.

The OB polypeptide, or functionally active fragment thereof, or an antagonist thereof, can be administered orally or parenterally, preferably parenterally. metabolic homeostasis is a continuous process, controlled release administration of ob polypeptide is preferred. For example, the polypeptide may be administered using 5 intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes. or other modes of administration. In one embodiment, a pump may be used [Langer et al., eds., Medical Applications of Controlled Release, CRC Pres., Boca Raton, Florida (1974); Sefton, CRC Crit. Ref. Biomed. Eng., 14:201 (1987); Buchwald et al., Surgery, 88:507 (1980); Saudek et al., N. Engl. J. Med., 321:574 (1989)]. In 10 another embodiment, polymeric materials can be used [Langer, 1974, supra; Sefton, 1987, supra; Smolen et al., eds., Controlled Drug Bioavailability, Drug Product Design and Performance. Wiley. New York (1984): Ranger et al., J. Macromol. Sci. Rev. Macromol. Chem., 23:61 (1983); see also Levy et al., Science, 228:190 (1985); During et al., Ann. Neurol., 25:351 (1989); Howard et al., J. Neurosurg., 15 71:105 (1989)]. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose [see, e.g., Goodson, in Medical Applications of Controlled Release, vol. 2, pp. 115-138 (1984)]. Other controlled release systems are discussed in the review by Langer, Science, 249:1527-1533 (1990). In another 20 embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (see Langer, 1990 supra): Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In a further aspect, recombinant cells that have been transformed with the OB gene
25 and that express high levels of the polypeptide can be transplanted in a subject in need
of ob polypeptide. Preferably autologous cells transformed with OB are transplanted
to avoid rejection: alternatively, technology is available to shield non-autologous cells
that produce soluble factors within a polymer matrix that prevents immune
recognition and rejection.

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The OB polypeptide can be delivered by intravenous, intraarterial, intraperitoneal, intramuscular, or subcutaneous routes of administration. Alternatively, the OB polypeptide, properly formulated, can be administered by nasal or oral administration. A constant supply of OB can be ensured by providing a therapeutically effective dose 5 (i.e., a dose effective to induce metabolic changes in a subject) at the necessary intervals, e.g., daily, every 12 hours, etc. These parameters will depend on the severity of the disease condition being treated, other actions, such as diet modification, that are implemented, the weight, age, and sex of the subject, and other criteria, which can be readily determined according to standard good medical 10 practice by those of skill in the art.

Pharmaceutical Compositions

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. 25 Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See. e.g., Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

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Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (E.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in Modern Pharmaceutics, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include the protein (or chemically modified protein), and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatized proteins. Protein may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski et al., 1981, supra; Newmark et al., J. Appl. Biochem., 4:185-189 (1982). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3-6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the protein (or derivative) the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the 5 release will avoid the deleterious effects of the stomach environment, either by protection of the protein (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential.

Examples of the more common inert ingredients that are used as enteric coatings are

10 cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate

(HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit

L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and

Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended
for protection against the stomach. This can include sugar coatings, or coatings
which make the tablet easier to swallow. Capsules may consist of a hard shell (such
as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin
shell may be used. The shell material of cachets could be thick starch or other edible
paper. For pills, lozenges, molded tablets or tablet triturates, moist massing
techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

25 Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation)

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and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material.

These diluents could include carbohydrates, especially mannitol, α-lactose, anhydrous
lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may
be also be used as fillers including calcium triphosphate, magnesium carbonate and
sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STARx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage

form. Materials used as disintegrants include but are not limited to starch including
the commercial disintegrant based on starch, Explotab. Sodium starch glycolate,
Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin,
orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be
used. Another form of the disintegrants are the insoluble cationic exchange resins.

Powdered gums may be used as disintegrants and as binders and these can include
powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt
are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin.

20 Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be

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used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, tale, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the protein (or derivative) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms i.e., gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film-coated tablet; the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating

10 may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary Delivery

Also contemplated herein is pulmonary delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while 15 inhaling and traverses across the lung epithelial lining to the blood-stream. Other reports of this include Adjei et al., Pharmaceutical Research, 7(6):565-569 (1990); Adjei et al., International Journal of Pharmaceutics, 63:135-144 (1990) (leuprolide acetate): Braquet et al., Journal of Cardiovascular Pharmacology, 13(suppl. 5):143-146 (1989) (endothelin-1); Hubbard et al., Annals of Internal 20 Medicine, 3(3):206-212 (1989) (α1- antitrypsin); Smith et al., J. Clin. Invest., 84:1145-1146 (1989) (α1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colorado, (March 1990) (recombinant human growth hormone); Debs et al., J. Immunol., 140:3482-3488 (1988) and Platz et al., U.S. Patent No. 5,284,656 (granulocyte 25 colony stimulating factor). Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

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Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of protein (or derivative). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified protein may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

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Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise protein (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per ml of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the protein (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane.

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or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing protein (or derivative) and may also include a bulking 5 agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, ε.g., 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm, for most effective delivery to the distal lung.

Nasal Delivery

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Nasal delivery of the protein (or derivative) is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

15 Methods of Treatment, Methods of Preparing a Medicament

In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are provided. Conditions alleviated by or modulated by the administration of the present derivatives are those indicated above.

Dosages

20 For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain the proper dosage. Generally, for injection or infusion, dosage will be between 0.01 µg of biologically active protein/kg body weight, (calculating the mass of the protein alone, without chemical modification), and 10 mg/kg (based on the same). The dosing schedule may vary, depending on the circulation half-life of the protein or derivative used, whether

the polypeptide is delivered by bolus dose or continuous infusion, and the formulation

Administration with other compounds

For therapy associated with obesity, one may administer the present protein (or derivatives) in conjunction with one or more pharmaceutical compositions used for treating other clinical complications of obesity, such as those used for treatment of diabetes (e.g., insulin), high blood pressure, high cholesterol, and other adverse conditions incident to obesity. Also, other appetite suppressants may be co-administered, e.g., amphetamines. Administration may be simultaneous (for example, administration of a mixture of the present protein and insulin) or may be in seriatim.

Nucleic Acid-based Therapeutic Treatment

The OB gene could be introduced into human fat cells to develop gene therapy for obesity. Such therapy would be expected to decrease body weight. Conversely, introduction of antisense constructs into human fat cells would reduce the levels of active OB polypeptide and would be predicted to increase body adiposity.

In one embodiment, a gene encoding an *OB* polypeptide is introduced in vivo in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, adipose tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSVI) vector [Kaplitt et al., Molec. Cell. Neurosci., 2:320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., J. Clin. Invest., 90:626-630 (1992), and a defective adeno-associted virus vector

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[Samulski et al., J. Virol., 61:3096-3101 (1987); Samulski et al., J. Virol., 63:3822-3828 (1989)].

In another embodiment, the gene can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al., Cell, 33:153 (1983); Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., J. Virol., 62:1120 (1988); Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty et al.; and Kuo et al., Blood, 82:845 (1993).

Alternatively, the vector can be introduced in vivo by lipofection. For the past 10 decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids in vitro. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for in vivo transfection of a gene encoding a marker [Felgner et al., Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987); see Mackey et al., Proc. Natl. Acad. 15 Sci. USA, 85:8027-8031 (1988)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner et al., Science, 337:387-388 (1989)]. The use of lipofection to introduce exogenous genes into specific organs in vivo has certain practical advantages. Molecular targeting of liposomes to specific cells 20 represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey et al., 1988, supra). Targeted peptides, e.g., hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector in vivo as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, ϵ .g.. transfection, electroporation, microinjection,

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transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, e.g., Wu et al., J. Biol. Chem., 267:963-967 (1992); Wu et al., J. Biol. Chem., 263:14621-14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Agricultural Apolications

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The OB gene can also be isolated from domestic animals, and the corresponding OB polypeptide obtained thereby. In a specific example, infra, the probe derived from the murine OB gene hybridizes to corresponding homologous coding sequences from a large number of species of animals. As discussed for human therapies, recombinant proteins can also be prepared and administered to domestic animals. Administration of the polypeptide can be implemented to produce leaner food animals, such as beef cattle, swine, poultry, sheep, etc. Preferably, an autologous OB polypeptide is administered, although the invention contemplates administration of anti-autologous polypeptide as well. Since the OB polypeptide consists of approximately 160 amino acid residues, it may not be highly immunogenic. Thus, administration of non-autologous polypeptide may not result in an immune response.

Alternatively, the introduction of the cloned genes into transgenic domestic animals would allow one to potentially decrease body weight and adiposity by overexpressing an OB transgene. The simplest means of achieving this would be to target an OB transgene to fat using its own or another fat specific promoter.

Conversely, increases in body fat might be desirable in other circumstances such as for the development of Kobe beef or fatty liver to make foie gras. This could be accomplished by targeting an antisense OB transgene to fat, or by using gene knockout technology. Alternatively, where an increase in body weight at percentage of fat is desired, an inhibitor or antagonist of the OB polypeptide can be administered. Such inhibitors or antagonists include, but are not limited to, antibodies reactive with the polypeptide, and fragments of the polypeptide that bind but do not activate the OB receptor, i.e., antagonists of the OB polypeptide.

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Cosmetic Implications

The OB polypeptide has significant value for cosmetic use, in addition to the health benefits. In particular, since the OB polypeptides of the invention, including derivatives and agonist analogs thereof, are useful for modulation of the rate and quantity of fat cell deposition in an animal, they are useful for reducing unsightly fat tissue, e.g., fat deposits in the abdomen, hips, thighs, neck, and chin that do not necessarily amount to an obese condition, but which nevertheless detract from an individual's appearance. The fat reduction effect is thought to be accomplished, in part, by a reduction in appetite, i.e., a reduction in food intake, by an increase in basal metabolism, or both. Thus, the present OB polypeptide, or its derivatives or agonist analogs, is useful for administration to a subject to effect cosmetic changes in fat tissue deposits, whether by modulating fat deposition, reducing appetite, or both

In addition, the present compositions and methods may be used in conjunction with

15 various procedures, such as cosmetic surgeries designed to alter the overall

appearance of a body (e.g., liposuction or laser surgeries designed to reduce body

mass by aspirating or ablating fat tissue), exercise (especially running and weight

training), low fat diet, hypnosis, biofeedback, as examples of the ways one may

attempt to decrease the percentage of fat tissue and improve the appearance of the

20 body.

Accordingly, the present invention relates to a method for effecting cosmetic fat tissue modulation in an individual comprising administering a fat modulating amount of an OB polypeptide, or derivative or agonist analog thereof, to an individual who desires cosmetic fat tissue modulation to improve overall body appearance. In a particular aspect, the fat tissue modulation is a consequence of appetite suppression. Preferably, the fat tissue modulation is a reduction in fat tissue.

In a further embodiment, the invention relates to a method for effecting cosmetic fat tissue loss comprising combining a procedure for changing body appearance with administration of a fat modulating amount of an OB polypeptide. or derivative or agonist analog thereof, to an individual who desires cosmetic fat tissue modulation to improve overall body appearance.

The OB Receptor

5 Development of small molecule agonists and antagonists of the OB factor will be greatly facilitated by the isolation of its receptor. This can be accomplished by preparing active OB polypeptide and using it to screen an expression library using standard methodology. Receptor binding in the expression library can be tested by administering recombinant polypeptide prepared using either bacterial or mammalian expression vectors, and observing the effects of short term and continuous administration of the recombinant polypeptide on the cells of the expression library, or by directly detecting binding of OB polypeptide to the cells.

As it is presently believed that the OB receptor is likely to be located in the hypothalamus and perhaps liver, preferably cDNA libraries from these tissues will be constructed in standard expression cloning vectors. These cDNA clones would next be introduced into COS cells as pools and the resulting transformants would be screened with active ligand to identify COS cells expressing the OB receptor. Positive clones can then be isolated so as to recover the cloned receptor. The cloned receptor would be used in conjunction with the OB ligand (assuming it is a hormone) to develop the necessary components for screening of small molecule modulators of OB.

A particular assay system that is to be utilized in accordance with the present invention, is known as a receptor assay. In a receptor assay, the material to be assayed is appropriately labeled and then certain cellular test colonies are inoculated with a quantity of both the labeled and unlabeled material after which binding studies are conducted to determine the extent to which the labeled material binds to the cell receptors. In this way, differences in affinity between materials can be ascertained.

Accordingly, a purified quantity of the weight modulator may be radiolabeled and combined, for example, with antibodies or other inhibitors thereto, after which binding studies would be carried out. Solutions would then be prepared that contain various quantities of labeled and unlabeled uncombined weight modulator, and cell 5 samples would then be inoculated and thereafter incubated. The resulting cell monolayers are then washed, solubilized and then counted in a gamma counter for a length of time sufficient to yield a standard error of <5%. These data are then subjected to Scatchard analysis after which observations and conclusions regarding material activity can be drawn. While the foregoing is exemplary, it illustrates the manner in which a receptor assay may be performed and utilized, in the instance where the cellular binding ability of the assayed material may serve as a distinguishing characteristic. In turn, a receptor assay will be particularly useful in the identification of the specific receptors to the present modulators, such as the db receptor.

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15 A further assay useful and contemplated in accordance with the present invention is known as a "cis/trans" assay. Briefly, this assay employs two genetic constructs, one of which is typically a plasmid that continually expresses a particular receptor of interest when transfected into an appropriate cell line, and the second of which is a plasmid that expresses a reporter such as luciferase, under the control of a receptor/ligand complex. Thus, for example, if it is desired to evaluate a compound 20 as a ligand for a particular receptor, one of the plasmids would be a construct that results in expression of the receptor in the chosen cell line, while the second plasmid would possess a promoter linked to the luciferase gene in which the response element to the particular receptor is inserted. If the compound under test is an agonist for the 25 receptor, the ligand will complex with the receptor, and the resulting complex will bind the response element and initiate transcription of the luciferase gene. The resulting chemiluminescence is then measured photometrically, and dose response curves are obtained and compared to those of known ligands. The foregoing protocol is described in detail in U.S. Patent No. 4,981,784 and PCT International Publication No. WO 88/03168, for which purpose the artisan is referred.

Once a recombinant which expresses the OB receptor gene sequence is identified, the recombinant OB receptor can be analyzed. This is achieved by assays based on the physical or functional properties of the OB receptor, including radioactive labeling of the receptor followed by analysis by gel electrophoresis, immunoassay, ligand binding, etc. Furthermore, antibodies to the ob receptor could be generated as described above.

The structure of the OB receptor can be analyzed by various methods known in the art. Preferably, the structure of the various domains, particularly the OB binding site, is analyzed. Structural analysis can be performed by identifying sequence similarity with other known proteins, particular hormone and protein receptors. The degree of similarity (or homology) can provide a basis for predicting structure and function of the OB receptor, or a domain thereof. In a specific embodiment, sequence comparisons can be performed with sequences found in GenBank, using, for example, the FASTA and FASTP programs [Pearson et al., Proc. Natl. Acad. Sci. 15 USA, 85:2444-48 (1988)].

The protein sequence can be further characterized by a hydrophilicity analysis (e.g., Hopp et al., 1981, supra). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the OB receptor protein, which may in turn indicate extracytoplasmic, membrane binding, and intracytoplasmic regions.

20 Secondary structural analysis (e.g., Chou et al., 1974, supra) can also be done, to identify regions of the OB receptor that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, as well as open reading frame prediction and plotting, can also be accomplished using computer software programs available in the art.

25 By providing an abundant source of recombinant OB polypeptide, and the opportunity to isolate the OB receptor (i.e., the db gene product), the present invention enables

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quantitative structural determination of the active conformation of the OB polypeptide and the OB receptor, or domains thereof. In particular, enough material is provided for nuclear magnetic resonance (NMR), infrared (IR), Raman, and ultraviolet (UV), especially circular dichroism (CD), spectroscopic analysis. In particular NMR provides very powerful structural analysis of molecules in solution, which more closely approximates their native environment (Marion et al., 1983, supra: Bar et al., 1985, supra; Kimura et al., 1980, supra). Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, 1974, supra).

More preferably, co-crystals of OB polypeptide and OB receptor can be studied. Analysis of co-crystals provides detailed information about binding, which in turn allows for rational design of ligand agonists and antagonists. Computer modeling can also be used, especially in connection with NMR or X-ray methods [Fletterick et al., eds., Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1986)].

Identification and isolation of a gene encoding an OB receptor of the invention provides for expression of the receptor in quantities greater than can be isolated from natural sources, or in indicator cells that are specially engineered to indicate the activity of a receptor expressed after transfection or transformation of the cells. Accordingly, in addition to rational design of agonists and antagonists based on the structure of the OB polypeptide, the present invention contemplates an alternative method for identifying specific ligands of OB receptor using various screening assays known in the art.

25 The invention may be better understood by reference to the following Examples, which are intended to be exemplary of the invention and not limiting therof.

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EXAMPLE SECTION

The following outlines the method used to identify the genetic material that is exemplary of the present invention. This endeavor comprises four sequential steps:

A) Genetic Mapping, B) Physical Mapping, C) Candidate Gene Isolation, and D)

Mutation detection. Following confirmation that the murine gene in object was isolated (Step D), the homologous human gene was sought, and both the murine and human genes and putative proteins were characterized. The steps are summarized in greater detail, below.

A. Genetic Mapping

10 The ob mutation was segregated in genetic crosses, and standard linkage analysis was used to position the mutation relative to RFLPs (restriction fragment length polymorphisms). These data placed the OB gene in an ~ 5cM interval on proximal mouse chromosome 6. (5cM is a measurement of genetic distance corresponding to 5 apparent genetic crossovers per 100 animals.) A total of 771 informative meioses were generated and used in subsequent genetic mapping (Friedman et al., 1991, supra). The genetic loci that were mapped relative to OB were all previously published. The two closest RFLPs described were defined by probes derived from the carboxypeptidase and met oncogene genes.

The genetic resolution of the experiments described above was inadequate to clone

ob, principally because none of the genetic markers were in tight linkage. In order
to identify the requisite tightly linked RFLPs, additional probes were isolated and the
genetic cross was expanded. A method known as chromosome microdissection was
used to isolate random pieces of DNA from proximal mouse chromosome 6 [Bahary
et al., Mannnalian Genome, 4:511-515 (1993)]. Individual cloned probes were tested
for tight linkage to ob. On the basis of these studies one probe, D6Rck13, also
termed psd3, was selected for further analysis owing to its genetic proximity to OB.

This probe was used to genotype 835 ob progeny from interspecific and intersubspecific crosses, which indicated that D6Rck13 is nonrecombinant in all 835 animals as reported in Bahary et al. In the course of physical mapping, a new polymorphic marker was identified from a cosmid subclone derived from YAC 53A6.

5 This new marker was positioned between D6Rck13 and the OB gene and was used to genotype the additional 771 informative meioses from intraspecific intercross and backcross. A single animal #167 was identified to bear a recombination crossover between ob and D6Rck39. These studies indicated that D6Rck39/D6RcK13 is ~0.06 cM from ob. An additional probe, Pax4, was identified that was .12 cM proximal to ob. Pax4 was recombinant in two animals; #111 and #420. Pax4 is a pseudogene that was previously mapped to proximal mouse chromosome 6 by Gruss and coworkers [Gruss et al., Genomics, 11:424-434 (1991)]. On this basis, it was determined that the OB gene resides in the ~0.2cM interval between Pax4 and D6Rck13. This led to efforts to clone the interposing DNA in an effort to isolate

B. Physical Mapping

15 OB.

The cloning of the DNA in this interval made use of yeast artificial chromosomes (YACs), a relatively new cloning vector that allows the cloning of long stretches of contiguous DNA often more than one million base pairs in length.

Yeast artificial chromosomes were isolated using D6Rck13 and Pax4. This was accomplished by preparing purified DNA probes and using them to isolate the corresponding YACs. These YACs (#8, #16, #107 and #24) were isolated and initially characterized, and on the basis of the resulting analyses it was concluded that YAC 16 was the YAC that extended furthest distally, i.e., closest to ob. The key end of YAC #16 was then recovered, and it was determined that this end was closer to ob than Pax4. This end was termed 16M(+). This conclusion was reached because it was shown that this probe was not recombinant in animal #420 (as was Pax4). This end was sequenced and used to develop a PCR assay. This PCR assay was used to screen a YAC library. Four positive clones were isolated. Subsequent

characterization of these YACs by end-rescuing, restriction mapping, pulse field gel electrophoresis, and Southern blots with the genetic crosses determined that two of these YACs, adu and aad, were critical for subsequent studies. YAC aad is a 550 kB nonchimeric YAC which extended furthest distally. Therefore, the distal end of this YAC, aad(pICL) was used to complete the physical map. YAC adu is 370 kb nonchimeric YAC and its distal end, adu(+), was determined to be nonrecombinant in all the ab progeny of the genetic crosses including animals #111 and #167, suggesting that the OB gene might reside in this YAC.

A PCR assay for these two ends, aad(pICL) and adu(+) was developed and used for isolating more YACs and P1 clones to continue physical mapping. The important P1 clones isolated by this effort included 498, 499, 500 (isolated using a probe derived from aad(pICL)) and 322, 323 and 324 (using a probe from adu(+)).

In the meantime, YACs isolated by D6Rck13 (53A6, 25A8, 25A9, 25A10) were characterized. These studies determined that 53A6 extended furthest proximally 15 toward the aad YAC. The size of the gap between 53A6 and aad was determined, to be ~70 kB. The key end of 53A6, 53(pICL) was then used to screen three available YAC libraries and a P1 library. A critical P1 clone, 325, was isolated. This P1 clone overlapped with the P1 clones isolated by aad(pICL) as described above, and therefore served to close the gap between 53(pICL) and aad(pICL). As 20 a result, the whole contig, containing YACs and P1 clones, of -2.5 million base pairs in length, and spanning Pax4, 16M(+), adu(+), aad(pICL), 53(pICL), D6Rck39 and D6Rck13, was cloned. By carefully mapping the sites of recombination apparent in animal #111 and #167, it was concluded that OB was situated in a 400 kB interval. To provide a working DNA source for isolating the OB 25 gene, about 500 kB covering this nonrecombination region was isolated in a total of 24 Pl clones. These Pl clones, including 322 and 323, which later were found to be useful clones, were used for exon trapping.

The physical map of the portion of the chromosome carrying OB is shown in Figure 7A. Figure 7B represents the YAC contig. Figure 7C represents the Pl contig.

Isolation of Candidate Genes

The method used to isolate genes in this interval was exon trapping. This method used a commercial vector to identify exon DNA (i.e., coding sequences) by selecting for functional splice acceptor and donor sequences in genomic DNA introduced into a test construct. The DNA from these P1 clones were grown and subcloned into the exon trapping vector. These clones were short inserts cloned into a Bluescript vector. Each clone was PCR amplified with PCR primers corresponding to plasmid sequences that flanked the insert. The PCR amplification was performed directly on the bacteria that carried the plasmid. The reactions were set up using a Biomek robot. The PCR products were electrophoresed on a 1% agarose gel in TBE buffer that contained ethidium bromide. The exon trapping technique was modified to eliminate contaminating E. coli DNA from the P1 clones, and to screen out the abundant artifactual exons, which exceeded 80-90% of the putative exons trapped. The exon trapping vector includes HIV sequences; a short segment of these vector sequences corresponds to this artifact.

The exon trapping experiment was performed using various P1 clones. Exon trapping products were then amplified by PCR, selected, and sequenced. Sequences of putative "exons" were compared with those in Genbank using the Blast computer program. About fifteen exons were selected for further examination by RT-PCR, Northern analysis, and zoo blot for the presence of corresponding RNA or conserved sequences. Seven of the fifteen putative exons, 325-2, 323-9, 322-5, D1-F7, 1H3, and 2G7, were found to encode an RNA transcript. 325-2 is a testis specific gene; 323-8 and 323-9 are likely two exons from the same gene expressed mainly in brain and kidney. IH3 and 322-5 represent two low level brain transcripts. D1-F7 is an exon from a previously cloned gene, inosine monophosphate dehydrogenase (IMPDH), which has ubiquitous expression pattern. None of these genes appeared to encode OB. 2G7, which is the OB exon, is discussed further below.

After three unsuccessful efforts to exon trap the *OB* gene, another attempt was made by pooling DNA from all the P1s from the critical *OB* region. These included P1s: 258, 259, 322, 323, 324, 325, 498, 499, 500, 653, 654 and others. Thereafter P1s 258, 260, 322, 498 and 499 were subcloned into the exon trapping vector, and subsequently several plates were prepared with bacterial clones, each of which carried a putative exon. Approximately 192 clones representing putative *OB* candidates were obtained. As noted above, a consistent artifact such that many of the isolates contained two trapped exons derived from the vector was observed. Thus, clones were identified both by their size and by the fact that hybridization of DNA probes corresponding to this artifact hybridized to the corresponding bands on a Southern blot of the gel. In this way, 185 out of 192 clones were excluded from further evaluation. Exclusion of the artifacts on the basis of size alone was not possible, as this could have, in the end, led to exclusion of the exon corresponding to *OB*.

Thus, of the 192 exons, a total of seven exons were selected for further study.

Templates for sequencing the seven exons were prepared, and sequencing was performed. The sequences for the seven exons were analyzed and it was found that seven were identical and one was an apparent artifact. In particular, clone 1D12 contained the "HIV sequence," i.e., the artifact band. This left three exons for further analysis: 1F1, 2G7 and 1H3. 1F1 was eliminated because it mapped outside the critical region. PCR primers for both 1H3 and 2G7 were selected and synthesized.

The sequence of the exon on 2G7 was determined, and is shown in Figure 10 (SEQ ID NO:7). PCR primers for 2G7 were selected and synthesized. The portions of the sequence corresponding to the PCR primers are underlined. The primers used were:

25 5' CCA GGG CAG GAA AAT GTG (Tm = 60.0'C)
(SEQ ID NO:8)
3' CAT CCT GGA CTT TCT GGA TAG G (Tm = 60.0'C)
(SEO ID NO:9)

These primers amplified genome DNA with PCR conditions as follows: 25-30 cycles at 55°C annealing for 2', 72° extension for 2', 94°C denaturation for 1' in standard PCR buffer. These primers were also used to generate a labeled probe by including ³²P-dCTP in the PCR reaction with a corresponding reduction in the amount of cold dCTP.

A RT-PCR was performed on a variety of tissue RNAs and it was concluded that 2G7 was expressed exclusively in white fat among the tissues examined (Figure 11A). Thereafter, ³⁷P-labeled 2G7 was hybridized to a Northern blot of tissue RNAs (Figure 11B) and showed that its RNA was expressed at high level in fat tissue but was either not expressed or expressed at very low levels in all other tissues (where the signals may be the result of fat contaminating the tissue preparations). Ten µg of total RNA from each of the tissues listed was electrophoresed on an agarose gel with formaldehyde. The probe was hybridized to the blot at 65°C in a standard hybridization buffer, Rapid Hybe (Amersham). The size of the RNA was approximately 4.9 kB. At this point 2G7 was considered to be a viable candidate gene for OB and was analyzed further.

D. Mutation Detection

In order to confirm that 2G7 encoded the *OB* gene, it was necessary to demonstrate differences in the levels of RNA expression of the DNA sequence of this gene in mutant as compared to wild-type animals. Two separate mutations of the *ob* gene are available for study, C57BL/6J *ob/ob* (1J) and Ckc/Smj *ob/ob* (2J). These will be referred hereinafter as 1J and 2J, respectively. (Informal nomenclature is used to refer to the mouse strains studied. Throughout this specification and in the drawings, it will be understood that C57BL/6J refers to C57BL/6J +/+; CKC/smj refers to SM/Ckc-+^{Dac}-ob^{2J}/ob^{2J}). RNA was prepared from fat tissue that had been isolated from 1J, 2J, and control animals. Total RNA for each sample was treated with DNase and then reverse transcribed using oligo-dT as a primer and reverse transcriptase. The resulting single-stranded cDNA was then PCR amplified either with the 2G7 primers (conditions shown above)

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for the lower band or with commercially available actin primers for the upper band.

The RT-PCR products were run on a 1% agarose TBE gel that was stained with ethidium bromide (Figure 12A). Using RT-PCT it was found that while 2G7 mRNA was expressed in 1J and all the other control mice, it was completely missing in 2J mouse. No signal was detected after 30 cycles of amplification. This experiment provided direct evidence that 2G7 corresponded to an exon from the OB gene.

Since the 2J mutation is relatively recent and is maintained as a coisogenic strain, this result was the first available evidence that indicated that 2G7 is an exon from the OB gene. The mutation is likely located in the promoter region which leads to total abortion of mRNA synthesis. The presence of a signal in 1J mouse in this RT-PCR experiment suggested that 1J might carry a point mutation which does not result in a gross change in size of the RNA sample. In addition, 2G7 mRNA was absent, when tested by RT-PCR, from four additional 2J animals.

This result was confirmed on a Northern blot (Figure 12B). Fat cell RNA was prepared from each of the strains (C57Bl/6J, IJ, CKC/smj, and 2J). Ten μg of these RNAs were run out and blotted. The blot was probed with the 2G7 probe that was PCR-labeled, by amplification of the material, i.e., band, in Figure 11 using ¹²P-dCTP in the PCR reaction. Actin is a control for the amount of RNA loaded. The actin signal is fairly similar in all of the samples. The OB signal is absent in brain because the mRNA is specific to fat cells.

The results of the Northern analysis confirm that 2G7 specific RNA is absent in 2J mice. The ob RNA is absent in the CKC/smj ob/ob mice because in this obese mutant strain the gene is disrupted such that no RNA is made. In addition, the level of 2G7 RNA was increased ~10-20 fold in 1J as well as db/db fat. These results are compatible with the hypothesis that OB either encodes circulating hormone or is responsible for the generation of a signal from fat cells that modulates body weight. These results supported the conclusion that 2G7 is the OB gene and predicted that 1J

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mice have a point mutation, probably a nonsense mutation leading to a premature translation termination

These Northern results have been replicated using fat cell RNA preparations from four different 2J animals (Figure 13). In this assay, ap2 is a fat-specific transcript 5 that was used as a control much the same as actin in Figure 12B. There is no significance to the varying density of the ap2 band. ap2 was labeled by designing PCR primers form the published ap2 sequence. The RT-PCR products of fat cell RNA were then relabeled using the same protocol for PCR labeling. This analysis demonstrates the presence of OB mRNA in normal homozygous or heterozygous animals, and its absence from 2J mutant animals.

The mutation has been identified in 1J mice. The mutation is a C to T base change that results in a change of an arginine to an apparent premature stop codon at amino acid 108, and in all likelihood accounts for the 1J mutation (Figure 14) despite high level expression of the ob mRNA (see Figures 12 and 13, C57BL/6J ob/ob lanes).

More recently, Southern blots have been used to conclude that the 2J mutation is the result of a detectable DNA change at the 5' end of OB that appears to completely abolish RNA expression. The exact nature of this possible rearrangement remains to be determined.

A genomic Southern blot of DNA from the CKC/smj (SM/Ckc-+^{Dac}) and C57BL/6J

20 mice using four different restriction endonucleases was performed in order to
determine whether the mutant ob yielded a unique fragment pattern (Figure 15A).

Approximately 10 μg of DNA (derived from genomic DNA prepared from liver,
kidney, or spleen) was digested with the restriction enzyme indicated. The DNA was
then electrophoresed in a 1% agarose TBE gel. The DNA was transferred to an

25 imobilion membrane and hybridized to the PCR- labeled 2G7 probe. The key band
is the uppermost band in the Bg/II digest for the CKC/smj ob/ob (SM/Ckc-+^{Dac})

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 ob^2/ob^2) DNA. This band is of higher molecular weight than in the other strain, indicating a mutation in this strain.

Figure 15B is a Southern blot of a BgIII digest of genomic DNA from the progeny of an ob^{2j}/+ x ob^{2j}/+ cross. Some of the DNAs have only the upper band, some 5 only the lower band, and some have both bands. The animals with only the upper band are allo-obese, i.e., ob^{2j}/ob^{2j}. These data show that the polymorphism (i.e., mutation) shown in Figure 15A segregates in a genetic sense.

EXAMPLE 1: cDNA Cloning and Sequence Determination of OB

Using the labeled 2G7 PCR probe, a total of fifty mouse cDNA clones from a murine fat cell \(\lambda\)gt11 cDNA library (Clonetech 5'-STRETCH cDNA from testicular fat pads of Swiss mice, \(\pm\)ML3005b), and thirty cross hybridizing human cDNA clones from a human fat cell \(\lambda\)gt10 cDNA library (Clonetech 5'-STRETCH cDNA from abdomen \(\pm\)HL1108a) were isolated. Library screening was performed using the plaque lift procedure. The filters from the plaque lift were denatured using the autoclave method. The filters were hybridized in duplicate with the PCR-labeled 2G7 probe (Rapid Hybe buffer, 65°C, overnight). After a 2-4 hour prehybridization, the filters were washed in 2x SSC, 2\% SDS, twice for 30 minutes at 65°C and exposed to x-ray film. Duplicate positives were plaque purified. Plaque purified phage were PCR-amplified using commercially available vector primers, \(\ell\), \(\lambda\)gt11. The resulting PCR products corresponded to the cDNA insert for each phage with a small amount of vector sequence at either end. The bands were gel purified and sequenced using the ABI automated sequencer and the vector primers to probe the DNA polymerase.

The raw sequencing data were then manually examined base by base to correct
mishearing from the computer program. As the correct sequence became available,
the downstream primers were synthesized and used to continue sequencing. Such
experiments were repeated until each available cDNA clone was sequenced and
synthesized into a contig. To date, ~3000 base pairs from the 5' end of the mRNA

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has been compiled. One of the cDNA clones extended to the 5' end of the mRNA since its sequence was identical to that of the 5' RACE product of fat tissue RNA (data not shown).

The sequence data revealed that there is a 167 amino acid open reading frame (Figure 1). A Kozak translation initiation consensus sequence was present with an adenosine residue three bases upstream of the ATG. Two classes of cDNA were found differing by inclusion or exclusion of a single glutamine codon. This residue is found in a position immediately 3' to the splice acceptor of the 2G7 exon. Since the CAG codon of glutamine includes a possible AG splice acceptor sequence, it appears that there is slippage at the splice acceptor site with an apparent 3 base pairs deletion in a subset of the cDNA, as shown below.

gln ser val

(splice acceptor site)

15

20 The "ag" in the sequences above corresponds to the assumed intron sequence upstream of the glutamine codon, and AG is the putative alternative splice site. This glutamine residue is located in a highly conserved region of the molecule and its importance for biological activity is as yet unknown.

A putative N-terminal signal sequence was detected, the signal cleavage site of which is predicted to be carboxy-terminal to the alanine residue at amino acid position 21. This putative signal sequence was confirmed by application of a computer algorithm to the method of von Heijne. Using this technique, the most probable signal sequence was identified in the polypeptide coding region corresponding to amino acids 1-23, having the sequence:

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MCWRPLCRFLWLWSYLSYVOA † VP (SEO ID NO:10)

in which the arrow indicates the putative signal sequence cleavage site. The rest of the amino acid sequence was largely hydrophilic and did not have any notable structural motifs or membrane spanning domains other than the N-terminal signal sequence. Specifically, we did not find consensus sequences for N-linked glycosylation or dibasic amino acid sequences indicative of protein cleavage in the predicted processed protein (Sabatini et al., The metabolic basis of inherited disease, pp. 177-223, C.V. Scriver et al. eds., McGraw-Hill, New York). Data base searches using Blast and Block programs did not identify any homologous sequences.

Human fat tissue RNA was analyzed on Northern blots, RNA species of a similar size to the mouse ob gene was detected. Sequencing and analysis of cDNA clones revealed that human OB also encodes a 167 amino acid polypeptide (Figure 2A and B and Figure 3). Two classes of cDNA, with or without three base pair deletions, were found in human as well (Figure 6). The mouse and human OB genes were highly homologous in the predicted coding region, but had only 30% homology in the available 3' and 5' untranslated regions. An N-terminal signal sequence was also present in the human OB polypeptide. Comparison of the human and mouse OB polypeptide sequences showed that the two molecules share an overall 83% identity at the amino acid level (Figure 4). The N-termini of the mature proteins from both species share even higher homology, with only six conservative and three nonconservative amino acid substitutions among the N-terminal 100 amino acid residues.

Genomic DNA was isolated from mouse, rat, rabbit, vole, cat, cow, sheep, pig, human, chicken, eel, and Drosophila, and restriction digested with *EcoR1*. The digests were electrophoresed on 1% agarose TBE gel. DNA was then transferred to an immobilion membrane and probed with the PCR-labeled 2G7 probe. The filter was hybridized at 65°C and washed with 2x SSC, 0.2% SDS at 65°C twice for twenty minutes each wash. *i.e.*, there were two buffer changes. These data indicate that *OB*

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is conserved among vertebrates (Figure 16). Note in this regard that there is a 2+ signal in eel DNA: eel is a fish.

In summary, available evidence suggests that body weight and adiposity are physiologically controlled. Seven years ago efforts began to identify two of the key components of this system: the OB and DB genes. As shown in this example, the OB gene has now been identified as a fat specific gene that plays a key role in regulating body weight. The product of this gene, which is most probably a secreted hormone, will have important implications for the diagnosis and treatment of nutritional disorders in man and non-human animals.

EXAMPLE 2: Expression of OB In Bacteria

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Both murine and human cDNAs encoding ob have been cloned into a pET-15b expression vector (Novagen). This vector contains a T7 promoter in conjunction with a lac operator, and expresses a fusion protein containing a histidine tag (His-tag) and a thrombin cleavage site immediately upstream of the coding sequence insertion site 15 (Figure 17) (SEO ID NOS:11 and 12).

The mouse and human cDNAs were modified such that the alanine at the end of the signal sequence was turned into an NdeI site, as was a separate sequence in the 3' region. Insertion of the NdeI site was accomplished using PCR with novel primers: Mnde-5' (murine five prime primer):

20 CTTATGTTCA TATGGTGCCG ATCCAGAAAG TC (SEQ ID NO:13)

Mnde-3' (murine three prime primer): TCCCTCTACA TATGTCTTGG GAGCCTGGTG GC (SEQ ID NO:14)

Hnde-5' (human five prime primer):
TCTATGTCCA TATGGTGCCG ATCCAAAAAG TC (SEO ID NO:15)

25 Hnde-3' (human three prime primer): TTCCTTCCCA TATGGTACTC CTTGCAGGAA GA (SEQ ID NO:16) The primers contain a 6-base pair mismatch in the middle that introduces Ndel restriction sites at each end of the PCR fragment. Phage carrying either the mouse or human cDNA were PCR amplified using those primers. The PCR product was digested with Ndel and gel purified on a 1% low melting point agarose gel. The gel purified bands were subcloned into the pET vector. The resulting plasmids were sequenced to ensure that mutations were not introduced during the PCR amplification step of cloning. Constructs for the human and murine cDNA that encode and that lacks glutamine 49 have been prepared. In particular, pET 15b constructs containing either the human or the mouse OB coding sequence, minus signal sequence and fused to a His-tag, have been made using a PCR cloning method. The constructs have been sequenced to ensure no sequence errors were introduced into the coding region of the OB sene during the PCR amplification step.

Two resultant plasmid constructs, pETM9 and pETH14, were selected to transform a bacterial expression host. Upon induction with 1 mM IPTG under optimal conditions, the transformed bacteria were able to produce 100-300 µg/ml of the OB fusion. The majority of the OB fusion protein was found in the inclusion body. After solubilization with 6M guanidine-HCl or urea, the fusion protein was purified through a His-binding (Ni-chelation) resin column. The conditions for column purification of the OB fusion protein (including binding, washing, and eluting) were established experimentally. The OB fusion protein binds to the resin at 5 mM imidazole/6M guanidine-HCl and stays bound at up to 20 mM imidazole/6M guanidine-HCl. The protein can be eluted from the resin at 60 mM imidazol/6M guanidine (Figure 18A,B). Both the purified human and mouse OB fusion proteins were further dialyzed in PBS to remove guanidine-HCl from the preparation, then used to raise polyclonal antibodies.

In order to test the biological activity of the fusion protein products, the refolding conditions for the purified protein were tested and developed. This involves initial dialysis of the fusion protein in a 1 M guanidine solution, followed by dilution with a 0.4 M arginine solution. The His-tag was removed from the fusion proteins before

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assaying for biological function. The tag removal was achieved by treating the fusion protein with thrombin from human placenta.

In addition, human and mouse OB gene coding sequences minus the signal sequence are each being inserted into a pET 12c vector using PCR cloning method. These constructs can direct the synthesized OB fusion proteins into the periplasmic space of the bacterial host cell. The OB fusion protein recovered from the periplasmic space may only need a simple gel filtration to be purified from other host proteins and will not be denatured during such a process.

EXAMPLE 3: Preparation of Antibodies to the OB Polypeptide

In addition to use of the recombinant protein to generate polyclonal antibodies, a set of four peptide sequences from the deduced murine OB sequence were identified using immunogenicity plot software (GCG Package). The four carboxyl terminal peptide fragments are:

15 (SEO ID NO:18):

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Val-Pro-Ile-Gln-Lys-Val-Gln-Asp-Asp-Thr-Lys-Thr-Leu-Ile-Lys-Thr

(SEO ID NO:19):

Leu-His-Pro-Ile-Leu-Ser-Leu-Ser-Lys-Met-Asp-Gln-Thr-Leu-Ala

(SEO ID NO:20):

20 Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gin-Thr-Ser-Gly-Leu-Gin-Lys-Pro-Glu-Ser-Leu-Asp

(SEO ID NO:21):

Ser-Arg-Leu-Gin-Gly-Ser-Leu-Gin-Asp-Ile-Leu-Gin-Gin-Leu-Asp-Val-Ser-Pro-Glu-Cys

These peptides were conjugated to KLH, and the peptide-KLH conjugates were used to immunize rabbits using standard techniques. Polyclonal antisera specific for each peptide is recovered from the rabbits.

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EXAMPLE 4: In Vitro Translocation of an OB Polypeptide

In order to confirm the presence of a functional signal sequence, a human cDNA that included the entire open reading frame was subcloned into the pGEM vector. Only the human cDNA was used in this experiment because suitable mouse subclones were 5 not recovered. Positive strand human ob RNA was transcribed using Sp6 polymerase and used in an in vitro translation reaction with and without canine pancreatic microsomal membranes. The primary translation product migrated with an apparent molecular weight of ~18 kD, which is consistent with that predicted by the cDNA sequence. Inclusion of the microsomal membranes in the reaction inhibited the 10 overall efficiency of translation - 5-fold. Nevertheless, approximately 50-70% of the OB primary translation product was truncated by approximately 2 kD in the presence of the membrane preparation, suggesting that the signal sequence is functional (Figure 19A). The size of the primary translation product of interleukin-1\alpha RNA, which does not encode a signal sequence, was unchanged when microsomal membranes were 15 included in the reaction. In order to confirm that translocation of the OB protein had taken place, the in vitro translation products were treated with Proteinase-K. Protease treatment resulted in the complete proteolysis of the 18 kD primary translation product while the 16 kD processed form was unaffected by the enzyme treatment, indicating that it had translocated into the lumen of the microsomes (Figure 19B). 20 These data are compatible with the hypothesis that OB is a secreted molecule.

After signal sequence cleavage, two cysteine residues would remain within the predicted protein raising the possibility that the molecule contains a disulfide bond characteristic of other secreted polypeptides [Shen et al., Science, 224:168-171 (1984)].

EXAMPLE 5: Characterization of the OB Gene

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To establish the relationship between obesity and genetic alterations in the OB gene in humans, the sequence of the human OB gene was determined (Figure 20A through

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C) (SEQ ID NOS:22 and 24). Specific primers from the human coding sequence were used to screen a human P1 library. Three different P1 clones were obtained, grown up, and PCR amplified using primers flanking the splicing site between the first and second coding exons. The entire intron region, around 2 kb, was amplified and partially sequenced (see Figure 20A; and as indicated in SEQ ID NO:22 and 24).

The gene structure of both the murine and human genes was characterized using PCR assays and other standard techniques. The mouse OB gene was found to consist of three exons, the second and third of which account for the coding sequence (Figure 20D). The coding region of the human OB gene shares the same structure; however, the human gene lacks a 5' exon and intron (Figure 20E).

Two sets of primers generated from the intronic sequences of the human gene have been prepared (Figure 20A through C). The sequences of the primers follows (F and R refer to forward and reverse, respectively):

HOB 1gF 5'-CCCAAGAAGCCCATCCTG-3' (SEQ ID NO:29)

15 HOB 1gR 5'-GACTATCTGGGTCCAGTGCC-3' (SEQ ID NO:30)

HOB 2gF 5'-CCACATGCTGAGCACTTGTT-3' (SEQ ID NO:31)

HOB 2gR 5'-CTTCAATCCTGGAGATACCTGG-3' (SEO ID NO:32)

DNA samples have been obtained from various sources, and these sets of primers are being used to amplify human genomic DNA from severely obese people. The PCR products were run on a low melting point agarose gel, and the bands were cut out and digested with agarase. The sequences were obtained using the ABI 373A DNA sequencer and Taq dideoxy terminator kit (ABI, Perkin-Elmer). One point mutation in an ob gene from a patient sample has been detected to date. This mutation is in the first exon and does not change the amino acid sequence. Preliminary data indicate that an insertion sequence may be present in the first exon of another patient.

A different automated sequencing method using Sequenase instead of Taq DNA polymerase may be employed to yield more easily readable sequences for mutation detection.

EXAMPLE 6: Expression of OB in Yeast

5 Following the positional cloning of OB, it became important to uncover the physiological mechanism by which the OB protein reduces food intake and body weight. The first step in this direction was to recombinantly produce a functional protein using an expression system. In addition to the successful bacterial expression system, a yeast expression system was also selected. Yeast expression has several 10 attractive features for expressing OB. The most important is that biologically active eukaryotic proteins are more likely to be produced. The OB polypeptide is secreted by mammalian cells. Protein secretion is very similar for all eukaryotes, which means that the yeast secretory apparatus is much more similar to the mammalian secretory pathway than bacterial secretory pathways would be. In particular, protein modifications of OB seen in mammalian cells would likely also be seen in the expression through the yeast secretory system. In addition, protein folding is carried out in passage through the secretory apparatus and thus, delivering ob through the yeast secretory apparatus is likely to give a properly folded protein with native biological activity. This is significant for OB because the two cysteine residues may 20 form a disulfide bridge. In contrast to secretory pathways, the reducing environment of the cell cytoplasm prevents formation of disulfide bridges; and therefore it is essential that OB pass through the secretory pathway in order for this disulfide bond to form in vivo. Other advantages have to do with the ease and quickness of manipulating yeast, the availability of vectors and strains, and the vast experience in 25 yeast recombinant technology.

A Pichia pastoris expression system was chosen for four reasons: (1) it has higher levels of heterologous protein expression than other yeast systems such as S. cerevisiae; (2) protein glycosylation is more similar to the mammalian system in P.

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pastoris than in S. cerevisiae (although glycosylation sites were not detected in ob using a computer search, there still remained the possibility of glycosylation at unrecognized sites); (3) P. pastoris secretes very few proteins natively, and thus it is generally straightforward to purify the expressed foreign protein; and (4) the 5 vectors and yeast strains are commercially available (from Invitrogen). Two strategies for generating yeast expression vectors are shown in Figures 21 and 22.

The vector chosen was pPIC.9. This vector contains a cloning site just downstream of the a-mating factor prepro coding sequence which directs the protein encoded by the gene cloned into the cloning site to be secreted by the secretory pathway. The 10 other important feature of the vector is a HIS4 gene that allows selection for uptake of the vector using a yeast auxotrophic strain grown on histidine-deficient media following transformation of the yeast with the vector. The cloning strategy was as follows: PCR amplify OB cDNA using a 5' primer that contains at its 3' end, sequence complementary to the sequence of OB just following the predicted leader 15 peptide cleavage site, and at its most 5' end, a sequence complementary to the 3' end of the \alpha-mating factor sequence of the vector. The 5' primer also contains an XhoI site. The 3' primer was designed to have at its 3' end a sequence complementary to the last few amino acids of OB and an EcoRI site at its 5' end. Following PCR amplification, the PCR product was digested with XhoI and EcoRI and cloned into similarly digested pPIC.9. Following the cloning of both the mouse and human OB cDNAs, each with and without the glutamine at codon 49, individual clones were isolated for all four constructs and sequenced to verify that the constructs were cloned in the correct orientation, and frame, and contained no mutations from the PCR amplification step. Following identification of clones with the correct sequence, these were transformed into P. pastoris strain GS115, a histidine auxotroph.

For the two mouse OB constructs, transformed yeast clones were screened for protein expression. As evidence that the transformed yeast contain OB, a DNA dot-blot assay and a colony hybridization assay were done which both showed OB sequence within the transformed yeast, but not within the untransformed yeast. Furthermore,

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the transformed yeast now secreted a 16 kDa protein into the culture media, whereas the untransformed yeast does not secrete a protein of this size (Figure 23A). This is the predicted size of OB. Individual clones for both mouse constructs have been identified that are high expressors for OB, and currently a purification strategy is being developed to purify ob to homogeneity. One strategy has been to purify OB on a cation exchange column (Figure 23B); preliminary data suggest that a strong cation exchanger may be useful. However, after cation exchange chromatography, the putative ob product is lost. This indicates the presence of a protease in the sample.

10 One strategy to overcome this problem is to prepare ob-His-tag fusions for expression in yeast (Figure 22). Further evaluation has demonstrated that OB without a His-tag associates tightly with a Ni-chelation column. Purification of the OB polypeptide by Ni-chelation, followed by gel filtration, yielded a product of sufficient purity for mass spectral analysis. Mass spec. confirms the molecular weight of the expressed protein is identical to the expected molecular weight, which strongly confirms that OB has been successfully expressed in Pichia.

However, the Ni-chelation/gel filtration purification protocol does not yield an OB polypeptide in sufficiently pure form. Additional small molecules are present. It does appear that the proteolytic activity elutes from the Ni-chelation column in the void volume. Accordingly, a three-step purification process is planned: Ni-chelation, followed by cation exchange (which eliminates the small molecule contaminants), followed by gel filtration.

Estimating expression level by Coomassie blue staining of SDS-PAGE gels reveals approximately 10 mg/l when yeast are grown in shaker flasks. These levels are expected to increase in fermentation vessels, and we are about to initiate fermentation with the hopes of obtaining larger quantities of protein. Regarding the human OB constructs, transformed yeast clones containing high copy numbers of the OB gene

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have been identified, and these are expected to express OB protein. As antibodies are developed, these will be used to confirm the identity of the secreted 16 kDa protein.

EXAMPLE 7: High Level Expression of an Ob Fusion Peptide in Bacteria

Preparation of freezer stocks:

- 5 To each of the two 4 ml aliquots of sterilized M9ZB media without the carbon source, 40 µl stock dextrose (0.4 g/ml, filter sterilized) 10µl ampicillin stock (200 mg/ml, and 5 µl chloramphenicol stock (34 mg/ml, in ethanol) were added. A single colony each of E. coli with cloned mouse and human OB1 DNA in a Novagen pET-14b vector was used to inoculate these. The tubes were incubated at 37°C overnight.
- 10 0.5 ml of the overnight cultures were used to inoculate 50 ml M9ZB media with dextrose, ampicillin and chloramphenicol. These were incubated at 30°C and the absorbance at 600 nm (A₆₀₀) was monitored periodically. At A₆₀₀ of about 1-1.2, 175 μl aliquots of the culture were mixed with 25 μl 60% glycerol in 2 ml eppendorf tubes, flash frozen in liquid nitrogen and stored at -80°C.

15 Culture growth:

50 ml M9ZB media with 0.5 ml 40% dextrose, 125 μl ampicillin stock and 50 μl chloramphenicol stock was inoculated with 1 ml freezer stock and incubated at 30°C. At A₆₀₀ of 1-1.2, 10 ml of this culture was used to inoculate each of four 2 L flasks with 500 ml M9ZB media with dextrose, ampicillin and chloramphenicol. These were incubated at 30°C until induction at A₆₀₀ of about 1-1.2 with a final concentration of 0.5 mM IPTG. The cultures were incubated overnight. The cells were harvested by centrifugation at 4000 rpm for 20 minutes. This expression system yield a recombinant OB polypeptide as a fairly high percentage of total protein; on the order of gram/liter of E. coli.

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Cell lysis and resuspension of inclusion bodies:

Cell paste was resuspended in a minimal volume of 20 mM HEPES, pH 7.2, 10% glycerol, 0.1 M KCI, 5 mM MgCl₂, 1% aprotinin, 1 mM PMSF, 5 µg/ml leupeptin and 50 µg/ml DNase I. The suspension was freeze thawed three times using liquid nitrogen and lukewarm water. Lysed cells were centrifuged at 18000 rpm for 30 minutes and resuspended in 20 mM HEPES, pH 7.5, 0.1 M NaCl. The suspension was sonicated and Triton X100 was added to a final concentration of 2%. This was centrifuged for 15 minutes at 18000 rpm. After two more such cycles, three cycles of Triton free washes were given. Finally the pellet was dissolved in 6 M GdHCl (guanidine-HCl), 20 mM HEPES, pH 7.5 by sonication followed by centrifugation. The supernatant was used for further purification.

The OB protein was purified in the unfolded state by immobilized metal ion affinity chromatography (IMAC). The solution was applied to a 40 ml column of Pharmacia chelating fast flow sepharose charged by 5 column volumes of 50 mM NiSO, and equilibrated in 6 M GdHCl, 20 mM HEPES, pH 7.5. The column was washed with 6 M GdHCl, 30 mM imidazole, 20 mM HEPES, pH 7.5. Finally, the protein was eluted with the same buffer containing 0.2 M imidazole. Unfolded protein in 6 M GdHCl was stored at 4°C after adding sodium acetate (NaAc) to 10 mM and adjusting the pH to about 4.5 with acetic acid.

- 20 Refolding and the purification of the protein:
 - 6 M GdHCl solution containing 100 mg protein was treated with 67 µl 1 M dithiothreitol (DTT) and diluted to about 67 ml with 6 M GdHCl, 10 mM NaAc, pH 4.5. It was left stirring at room temperature for about an hour. It was then diluted into 4 L of 20% glycerol, 2.5 mM CaCl₂, 20 mM Tris, pH 8.4 buffer with stirring.
- After proper mixing, the solution was left at room temperature for about 8 hours without further stirring. Then 2000 units of purified bovine thrombin (from thrombostat, a Parke-Davis product) was added, and the solution was left with gentle stirring. After 2.5 hours it was redosed with 2000 units of thrombin and the cleavage of the histidine-tag was continued for 3 more hours. The thrombin cleavage was

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arrested by adding PMSF to a final concentration of 0.1 mM. The solution was filtered and stored at 4°C.

The cleaved protein was further purified on the same IMAC column as above, equilibrated in 1 M KCl, 20% glycerol, 20 mM HEPES, pH 8.4 buffer. After 5 loading the protein solution, it was washed with the same buffer and the cleaved protein was eluted with 1M KCl, 20 % glycerol, 40 mM imidazole, 20 mM HEPES, pH 8.4. Uncleaved protein eluted at 0.2 M imidazole.

Purified cleaved protein was concentrated, treated with 50-100 mM EDTA, 10 mM potassium ferricyanide (to complete any incomplete oxidation) and gel filtered on a superdex 75 16/60 column. Yields using this procedure approached 50% of the starting peptide.

Once purified, the expressed protein has been characterized by several methods. Physical characterization includes dynamic light-scattering to determine homogeneity of structure and is used as a measure of proper folding. Light scattering data indicate that the human OB polypeptide is expressed predominantly or exclusively as a monomer, while the murine OB polypeptide can be found as a dimer as well as a monomer.

Assays with Ellman's reagent and mass spectroscopic analysis confirm that the cyteine residues form a disulfide bond in the protein. This oxidized form of the polypeptide was administered to mice, as described infra, and demonstrated biological activity.

Circular dichroism has been used to roughly determine the structural geometry of the protein. CD spectra in a physiological buffer (pH about 8, approximately physiological ionic strength) indicate that the human OB polypeptide has about 60% \(\alpha\)-helical structure and about 40% random coil structure. The murine OB polypeptide was found to have about 50% \(\alpha\)-helix and 50% random coil by CD spectroscopy.

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Limited proteolysis, followed by mass spectrometry (Cohen et al., 1995, supra) has been employed to identify portions of the OB polypeptide that are accessible to proteolysis. This analysis has demonstrated the presence of a flexible loop structure of amino acid residues 54 to 60 (as depicted in Figure 4). It is likely that this flexible loop connects two domains of defined 2° structure, e.g., a-helix.

Importantly, as shown in the following Examples, bioactivity of the purified protein was assayed by administering the protein to both lean and obese rodents via an osmotic pump (e.g., an ALZET osmotic pump from Alza Corporation, Palo Alto, CA) or by daily bolus dose i.p. over at least a two-week period and effects on feeding behavior and body weight were observed.

EXAMPLE 8: Weight Reducing Effects of the OB Polypeptide (Leptin)

The gene product of the mouse OB locus plays an important role in regulating body weight. The present Example establishes that the OB protein circulates in mouse, rat and human plasma. The circulating form in all three species has an identical molecular weight by SDS-PAGE to the deduced polypeptide sequence without the signal sequence, suggesting that, in vivo, the protein is not processed after cleavage of the signal sequence. The OB protein was absent in plasma from C57/Bl6J ob/ob mice and present at ten-fold higher concentrations in plasma of db/db mice and twenty-fold higher levels in plasma of falfa rats relative to controls. It is suggested that these obese animal mutants are resistant to the effects of OB. There were seven-fold differences in plasma levels of the OB protein within a group of six lean human subjects. Daily injections of the recombinant mouse OB protein dramatically reduced body mass in ob/ob mice, had significant effects on body weight of wild-type mice but had no effect on db/db mice. These data suggest that the gene product of the OB locus serves an endocrine function to regulate body weight.

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Materials and Methods

Rabbits were immunized with recombinant protein in Freund's adjuvant (HRP, Inc.). Immunopurified anti-mouse OB antibodies were prepared by passage of antiserum over a sepharose 4B column conjugated to the recombinant protein as described 5 [Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988)]. Immunoprecipitation of mouse plasma was carried out as follows: 0.5 ml of plasma from mouse, rat and human containing approximately 2.5 mM EDTA was pre-cleared with unconjugated sepharose-4B at room temperature with rocking for 2 hours. The sepharose was removed by spinning 10 and 50 ml of a 50% slurry of antibody-conjugated sepharose containing affinitypurified antibody at a concentration of 1 mg/ml of packed sepharose was added. One-half ml of 2x RIPA buffer was added to give final binding conditions as follows: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and 0.025% sodium azide. The reaction was carried out overnight at 4°C with rocking. The antibody-conjugated sepharose was washed 8 times using RIPA buffer, followed by rinsing three times with PBS, and run on a 15% SDS-PAGE. The proteins were transferred to nitrocellulose and Western blotted with a biotinylated immunopurified antibody against the recombinant protein. The secondary antibody used was HRP-streptavidin and ECL was used for detection.

To quantitate the amount of OB in mouse serum, increasing amounts of the refolded recombinant mouse OB protein (0.01, 0.1, 0.5, 2.0, 15.0 ng) were added to 100 λ of C57BL/6J ob/ob plasma and incubated at 4°C for 3 hours with the protein A sepharose conjugated antibody. After extensive washing with buffer A (10 mM sodium phosphate buffer, pH 7.4; 100 mM NaCl; 1% Triton X-100, 5 mM EDTA, 1 mM PMSF), samples were resuspended in sample buffer, loaded on a 15% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was performed using an immunopurified biotinylated anti-amino-terminus antibody as a primary antibody and HRP-streptavidin as a secondary antibody, followed by ECL detection.

Cytoplasmic extracts were prepared by homogenizing adipose tissue in NDS buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 60 mM ICCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β -mercaptoethanol, 0.5 m EGTA, 2 mM EDTA, 0.5 % NP-40) by polytron and dounce homogenization, and removal of nuclei was accomplished by centrifuging at 700 g.

Immunoprecipitations were performed as described above except that immunopurified anti-human OB antibodies were used. For the ELISA, 100 ml of a 1 mg/ml solution of immunopurified anti-human OB antibody was dissolved in a borate buffered PBS solution and applied overnight to microtiter (Corning cat. #2595) plates at 4°C. The 10 plates were then washed 4 times with borate saline solution containing 0.05% Tween 20 and excess liquid was removed. Plates were blocked by incubation at room temperature for 2 hours with 240 ml per well of borate saline buffer containing 0.3 % gelatin and then washed and dried. Either known amounts of a refolded human OB protein or plasma samples in a 100 ml volume were incubated in individual wells 15 overnight at 4°C. After washing, the plates were incubated with 100 ml of a biotinylated immunopurified anti-human antibody (0.1 mg/ml in a gelatin-borate buffered solution) for 4 hours at room temperature. After washing, horse radish peroxidase (HRP)-Streptavidin was added to the plates (0.1 mg/ml in borate buffer, 0.3% gelatin). HRP substrate solution (ABTS, 0.3 mg/ml and H2O2, 0.01% in citric 20 acid) was then used for detection and the O.D. was measured at 414 nM to quantitate the antibody binding.

The mouse and human OB gene coding sequences were PCR amplified from plasmids containing OB cDNA sequences and subcloned into the pPIC.9 plasmid (Invitrogen). The human 5' primer used was

25 5' GTATCTCTCGAGAAAAGAGTGCCCATCCAAAAAGTCCAAG 3' (SEQ ID NO:34) and the 3' primer was 5' GCGCGAATTCTCAGCACCCAGGGCTGAGGTC 3' (SEQ ID NO:35). For mouse, the 5' primer was

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5' GTATCTCTCGAGAAAAGAGTGCCTATCCAGAAAGTCCAGG 3' (SEO ID NO:36)

and the 3' primer was

5' GCGCGAATTCTCAGCATTCAGGGCTAACATC 3' (SEQ ID NO:37).

5 The 5' primer for both mouse and human contains a XhoI site at the 5' end and coding sequences for the last 4 amino acids of the α -mating factor signal sequence present in the vector pPIC.9. This vector directs secretion of heterologously expressed genes from the cell into the culture media. The 5' PCR primer also includes the first 19 nucleotides of the OB gene open reading frame after the signal 10 sequence cleavage site, before the alanine at amino acid position 21. The 3' primer contains an EcoRI site at its 5' end, which is immediately followed by sequences complementary to the putative OB stop codon. The PCR conditions were as follows: denaturing for 1 min. at 94°C, annealing for 1 min. at 55°C and extension for 2.5 min. at 72°C. Low-cycle PCR (15 cycles) and the proof-reading polymerase PFU 15 (Stratagene) were used to limit the number of PCR-generated mutations. The PCR products were digested with XhoI and EcoRI and cloned into similarly digested vector, pPIC.9. All constructs were sequenced on both strands to ensure the absence of any PCR-generated mutations. Clones were transformed into Pichia pastoris (His') by the spheroplast method and selected on histidine deficient media. Approximately 20 200 mouse and human clones were screened for high-copy number integration by a colony hybridization assay. The high copy number clones were then assayed for OB expression, initially by Coomassie staining showing the presence of a novel 16 kD protein present in the culture media of transformed yeast. The 16 kD band was confirmed to be OB using antibodies raised against the bacterially expressed OB 25 protein. The recombinant proteins were purified by a two-step purification method described below. Mass spectrometry and cyanogen bromide treatment were performed as described in Beavis et al., Proc. Natl. Acad. Sci. USA, 87:6873-6877 (1990).

The entire OB coding sequence of the mouse and human OB genes C-terminal to the signal sequence were subcloned into the pET15b expression vector (Novagen) and overexpressed in Escherichia coli [BL21(DE3)plYsS] using the T7 RNA polymerase system [Studier et al., Meth. Enzymology, 185:80-89 (1990)]. Cells grown at 30°C 5 to an absorbency of 0.7 at 595 nM and induced with 0.5 mM isopropyl-β-Dthiogalacto-pyranoside overnight were collected by low-speed centrifugation. Lysis was performed by three cycles of freeze thaw and DNA digestion was perform with Membrane extraction was performed by sonication and detergent DNaseI. solubilization, and the final inclusion body pellet was dissolved in 6M guanidine-HCl, 10 20mM HEPES, pH8.4. Recombinant OB proteins were purified under denaturing conditions by IMAC using a Ni-ion affinity column and washing with increasing amounts of imidazole. Purified denatured OB protein was then stored in 6 M guanidine-HCl, 10 mM sodium acetate (NaAc), pH 5, and reduced using 1 mM DTT at room temperature for 1 hour. Denaturation was performed by diluting the reduced 15 protein into 20% glycerol, 5 mM CaCl₂, 5 mM NaAc, pH 5, through mixing and incubation at room temperature for 8-12 hours. After denaturation the pH was adjusted to 8.4 by addition of Tris to 10 mM, and the hexa-histidine tag was removed by thrombin cleavage. Cleaved, renatured protein was repurified by IMAC to separate product from thrombin and uncleaved fusion protein. Cleaved, renatured 20 protein elutes from the Ni-ion affinity column at 40 mM imidazole, whereas thrombin is not retained and uncleaved fusion protein elutes at 0.2 mM imidazole. Product was then concentrated, treated with 100 mM EDTA and 10 mM potassium ferricyanide and further purified by gel filtration using Pharmacia superdex 75 16/60 column.

An Ellman's assay was conducted as described in Ellman, Arch. Biochem. Biophys., 82:70-77 (1959). Ellman's reagent was prepared by dissolving 39.6 mg 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 10 ml 0.05 M phosphate, pH 8. A calibration curve was constructed in the concentration range of 10-120 mM free sulfhyd-yl (using a 1 mM stock solution of reduced DTT) at 412 nm. Each assay was performed using 0.02 ml Ellman's reagent and a total reaction mixture of 0.5 ml. The measured extinction coefficient was 12974 M'cm' for free sulfhydryl group (correlation

coefficient 0.99987), which is within 5% of the previously reported value of 13600 $M^{-1}cm^{-1}$.

Fifty ml of 2 mg/ml pure gel filtered protein, corresponding to a possible free sulfhydryl concentration of about 24 mM in the final reaction mixture, was subjected to Ellman's assay. The resulting solution gave A₄₁₂ of about 0.02, suggesting that the two cysteine residues in the protein are in an oxidized state forming cystine or that their free sulfhydryl groups are completely buried within the inaccessible core of the folded protein. Identical results were obtained by conducting the same assay on unfolded protein in the presence of 6 M guantidine-HCl.

Mice were individually caged in a pathogen-free environment and acclimated to a diet containing 35 % (w/w) Laboratory Rodent Diet 5001 (PMP Feeds, Inc.), 5.9% (w/w) tapioca pudding mix (General Foods) and 59.1% water, which has an energy content of 1.30 kcal/gm. The diet was sterilized by autoclave and packed into 60 mm plastic dishes, which were fixed to the tops of 100 mm petri dishes. Tapioca gives the diet 15 a pasty texture making it difficult for the animal to spread the food in the cage. The 100 mm lid recovers the small amount of food spilled by the animal. A fresh dish of food was placed into the cage each morning and the previous day's dish was removed and weighed. The difference in weight provided a measure of daily food consumption. Effects of recombinant protein on food intake and body weight were 20 measured in three strains of mice: C57Bl/6J ob/ob, C57 Bl/Ks db/db and CBA/J +/+, purchased from the Jackson Laboratory. Thirty mice from each strain were divided into groups of 10. One group from each strain received daily intraperitoneal (i.p.) injections of the refolded bacterial ob protein at a dose of 5 mg/g/day in 300 μl of PBS. A second group received i.p. injections of the same volume of PBS. 25 These control mice received injections of the PBS dialysate of the recombinant protein. The PBS was cleared of endotoxin using an Acticlean ETOX column. A third group of animals did not receive injections. Food intake was recorded daily and body weight measurements were recorded regularly over a 3.5 week interval. For

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the pair feeding experiment, the food intake of a separate group of ob mice was matched on a daily basis to that consumed by the ob mice receiving protein.

Results

The OB Protein Circulates in Mouse, Rat and Human Plasma.

5 Recombinant mouse and human OB protein was prepared using the pET 15b bacterial expression vector (Novagen) and by cloning into Pichia pastoris, a yeast expression system that secretes recombinant proteins directly into the culture media. The ob protein expressed in yeast includes the 146 amino acids carboxy-terminal to the signal sequence. Rabbits were immunized with the bacterial proteins (HRP, Inc.).
10 Antibodies were immunopurified (Research Genetics) and used for immunoprecipitations and Western blots of protein from plasma and adipose tissue.

The OB protein from mouse plasma migrates with an apparent molecular weight of 16 kD by SDS-PAGE. The electrophoretic mobility is identical to the recombinant OB protein secreted by yeast after signal sequence removal (Figure 24A) The protein was not detected in plasma from C57BL/6J ob/ob mice that have a nonsense mutation at codon 105. Several different antisera failed to identify the truncated 105 residue polypeptide chain predicted by the cDNA sequence.

A ten-fold increase in the level of circulating protein was observed in db/db mice relative to a control animal (Figure 24A). Immunoprecipitation of plasma from wild20 type and fa/fa rats revealed a twenty-fold increase in the level of OB protein in the mutant rat compared to wild type (Figure 24B). The db mutation results in an obese phenotype identical to that seen in ob mice (Bahary et al., 1990, supra). fatty rats are obese as a result of a recessive mutation in a gene homologous to db (Truett et al., 1991, supra). In order to quantitate the level of OB in mouse plasma, increasing amounts of recombinant protein were added to serum and immunoprecipitated (Figure 24C). A linear increase of the signal intensity on Western blots was seen with increasing amounts of recombinant protein. Comparison of the signal intensity of the native protein in mouse plasma to the standards indicated that the circulating level of

the OB protein in wild type mice is approximately 20 ng/ml. These data demonstrate that the immunoprecipitations and Western blots were performed under conditions of antibody excess. Increased levels of the OB protein were also seen in protein extracts of adipose tissue from db/db mice relative to controls (Figure 24D). As expected for a secreted protein, the protein from the adipose tissue fractionated with the crude membrane fraction (data not shown).

Plasma samples from six lean human subjects with a Body Mass Index less than 25 (BMI = weight/length²) were immunoprecipitated using immunopurified antibodies to the human protein. The immunoprecipitated material migrated with an electrophrotic 10 mobility identical to that seen for the 146 amino acid human protein expressed in yeast. The intensity of the signals varied significantly among the six samples (Figure 25A). Densitometry of the autoradiograph revealed an approximately five-fold difference in the levels in individuals HP1 and HP6, with intermediate levels in the other subjects. An enzyme linked immunoassay (ELISA) was developed using the immunopurified antibody and the refolded bacterial protein as a standard (see below). The resulting standard curve is shown in Figure 25B. Using this assay, the plasma levels of the OB protein in the six human plasma samples varied between 2-15 ng/ml (Figure 25C). The level of the OB protein in plasma from HP6 was outside of the linear range of the immunoassay and is \geq 15 ng/ml. These quantitative differences correlated with those seen on Western blots.

Preliminary data suggest that leptin may circulate, at least in part, complexed to another protein or proteins. This conclusion was based on heterogeneity of the shape of the titration curve for serum compared with recombinant standard. Analysis of a large amount of leptin immunopurified on a rabbit anti-OB column by gel filtration 25 HPLC under denaturing and non-denaturing conditions, with monitoring by ELISA and SDS-PAGE suggested that the OB polypeptide behaved like a high molecular weight complex. However, these data remain preliminary; the OB binding protein. if any, has yet to be characterized.

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Structural Features of the OB Protein.

Since the OB protein has two cysteine residues, it could form either intra- or intermolecular disulphide bonds under oxidizing conditions in vivo. Western blots were repeated with and without the addition of reducing agents to the sample buffer. 5 Under both conditions, the OB protein in human serum migrated as a monomer (data not shown). Under nonreducing conditions, protein immunoprecipitated from db mouse serum was detected at positions consistent with that of both a monomer of 16 kD and a dimer of approximately 32 kD (Figure 26A). The higher molecular weight mojety disappeared under reducing conditions suggesting that a fraction of mouse OB circulates as a higher molecular weight species via formation of an intermolecular disulphide bond. Approximately 80% of mouse OB circulates as the approximately 16 kD protein and 20% as the approximately 32 kD form.

The same molecular forms are seen when the mouse and human proteins are expressed in Pichia pastoris [Abrams et al., Immunol. Rev., :5-24 (1992)]. In these 15 studies, the DNA sequence corresponding to the 146 amino acid mature OB protein was cloned downstream of the yeast α-mating factor signal sequence in the pPIC.9 vector (Invitrogen). The OB protein was purified from the yeast media of strains expressing the mouse and human proteins and electrophoresed under reducing and nonreducing conditions (Figure 26A). The mouse protein was expressed in yeast mainly as a dimer under nonreducing conditions, and only as a monomer in the presence of reducing agents. The recombinant human protein migrated to the position of a monomer under both conditions (data not shown).

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The purified human protein expressed in Pichia had a molecular mass of 16,024±3 Da as determined by mass spectrometry 1990 (Beavis, 1990, supra). This value is in agreement with the mass calculated from the amino acid sequence of the protein containing a single intramolecular disulfide bridge (16,024 Da). Matrix-assisted laser desorption mass spectometric analysis of cyanogen bromide cleavage products of the protein indicates that cysteines 117 and 167 are linked through an intramolecular

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disulphide bond (Figure 26B) Cyanogen bromide cleaves carboxyterminal to methionine residues.

Preparation and Characterization of Bioactive Recombinant Protein.

Mouse OB protein was expressed in E. coli from a pET 15b plasmid as an insoluble fusion protein, with a 20 residue, N-terminal hexa-histidine tag containing a thrombin cleavage site. Bacterial inclusion bodies were solubilized using guanidine-HCl and purified under denaturing conditions using immobilized metal ion affinity chromatography (IMAC) (Figure 27). Purified, denatured fusion protein was reduced, diluted and permitted to refold in aqueous solution at room temperature.

10 Following thrombin cleavage, renatured mouse OB protein containing four additional N-terminal residues (Gly-Ser-His-Met; SEQ ID NO:38) was repurified by IMAC to >98% homogeneity, as judged by SDS-PAGE and mass spectrometry. Matrix-assisted laser desorption mass spectrometry gave a measured mass of 16,414±3 Da (predicted mass = 16,415 Da). Both reducing and non-reducing SDS-PAGE gels demonstrated a single molecular species with apparent and molecular weight of 16 kD (data not shown).

Dynamic light scattering using a DP801 Molecular Size Detector (Protein Solutions, Inc.) demonstrated that the renatured mouse OB protein was largely monomeric, with some higher-order aggregates. The protein was treated with EDTA and chemically oxidized. Higher molecular weight species were then removed by gel filtration. Further dynamic light scattering confirmed that the purified, renatured recombinant mouse OB protein was monodispersed. Following dialysis against phosphate buffered saline (PBS), bacterial endotoxin was removed using an Acticlean ETOX column (Sterogene Bioseparations, Inc.). The final yield of protein was 45 mg/l.

25 Ellman's assay was performed on the purified, renatured recombinant mouse OB protein to assess its oxidation state (Ellman, 1959, supra). Both renatured protein and protein unfolded by 6M guanidine-HCl demonstrated <0.5% free sulfhydryl content, demonstrating that the monomeric product contains an intramolecular</p>

disulphide bond. This was confirmed by mass spectrometry of the cyanogen bromide cleavage products of the refolded bacterial protein (data not shown).

Bioactivity of the OB Protein. The purified, renatured recombinant mouse OB protein was administered as a daily intraperitoneal injection of 5 mg/kg/day to groups of 10 5 C57Bl/6J ob/ob (age, 16 weeks), C57Bl/Ks db/db (age, 12 weeks) and CBA/J +/+ (age, 8 weeks) mice. An equal number of animals received PBS as a daily injection. The PBS used for the control injections was derived from the dialysate after equilibration of the protein. Ten additional animals from the three mouse strains did not receive injections. The food intake of individual animals was monitored daily and 10 the weights of the animals were recorded at three or four day intervals. The cumulative results for food intake and body weight from each of the 9 groups of mice are shown in Figure 28A through F, and the statistical significance of the data are shown in Table 1. The food intake of the C57Bl6J ob/ob mice injected with protein was significantly decreased after the first injection and continued to decrease until the 15 fifth day, when it stabilized at a level equal to approximately 40% of the intake of the animals receiving injections of PBS (p < .001). The sham injected OB mice did not lose weight over the three week study period. The C57Bl/6J ob/ob mice receiving protein lost approximately 10% of their body weight after 5 days (p < .001). These animals continued to lose weight over the three week treatment at which point the weight of the ob animals receiving protein had decreased to an average of 60% of their initial body weight (p < .0001). A separate group of ob mice were pair fed to the ob mice receiving protein. The data in Figure 29B show that the pair fed mice lost significantly less weight than the animals receiving the recombinant protein (p < .02). A photograph of two mice receiving injections of either protein or vehicle shows the gross difference in appearance resulting from the protein treatment (Figure 29B). In order to further ascertain the effects of the protein, autopsies of two mice in each of the groups were performed. Gross inspection of the ob mice receiving protein revealed a dramatic decrease in body fat as well as the size of the liver. The liver weights of the db and wild-type mice were unchanged with treatment. The livers from the ob mice receiving the injections of PBS weighed 5.04 and 5.02 grams vs. 2.23 and 2.03 grams in the animals receiving the recombinant protein. In contrast to the pale fatty liver characteristic of ob mice, the liver from the ob mice receiving protein acquired the darker color characteristic of normal liver (Figure 29C). Histologic sections of the liver indicated that the untreated animals had a fatty
liver that was markedly improved in protein treated animals (data not shown).

In contrast to the ob mice, there were no significant differences in body weight or food intake in the C57BL/Ks db/db mice receiving protein relative to the control group receiving vehicle (Figure 28A through F, Table 1). All three groups of db/db mice lost between 2-5 grams during the treatment period. The average blood glucose of the db mice was measured using a glucometer, and was ≥ 500 mg/dl in all of the mice indicating that these animals had developed diabetes secondary to obesity. The injections of db mice were terminated after two weeks.

In wild-type mice there was a small but significant decrease in body weight following administration of the recombinant ob protein (Figure 28A-F, Table 1). After five days of protein injection, the treated mice lost an average of 0.5 grams while control mice gained 0.4 grams (p < .02). At two subsequent time points the animals receiving protein weighed significantly less than the mice receiving daily injections of PBS. The significance of the weight change was reduced at the later time points. In the animals that lost weight, the food intake was not significantly different from control animals. The injections of PBS had a small but significant effect on food intake and body weight in ob, db and wild-type mice as compared to mice not receiving injections (p < .05).

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TABLE I

		WEIGHT	CHAN	GE		
Animal Group	Treatment Group	Days	n_	Meso	Std. Error	P
ob/ob	protein vehicle	1-5	10 9	-6.38000000 -0.1444444	0.47628190 0.24444444	<0.001
	protein vehicle	1-12	10 9	-14.45000000 0.98888889	0.70793126 0.38058597	< 0.001
	protein vehicle	1-27	6 5	-24.28333333 4.30000000	0.69924563 0.79874902	< 0.0001
db/db	protein vehicle	1-5	10 10	-1.47000000 -2.00000000	0.36939891 0.23142073	0.240
	protein vehicle	1-12	10 10	-3.75000000 -4.19000000	0.77348418 0.34655447	0.610
CBA/J	protein vehicle	1-5	10 10	-0.48000000 0.38000000	0.17876117 0.21489015	0.006
	protein vehicle	1-12	10 10	-0.12000000 1.20000000	0.45748103 0.18378732	0.015
	protein vehicle	1-27	5	1.9 800000 0 2.23333333	0.48723711 0.20763215	< 0.651

Discussion

An endocrine function for the protein product of the OB locus was first suggested by

5 Coleman, who showed that the body weight of ob/ob mice was reduced after
parabiotic union to normal or db mice (Coleman et al., 1978, supra). The results
indicated above support this hypothesis by showing that OB protein circulates in the
bloodstream and that injections of recombinant protein reduce body weight. The
molecular weight of the gene product encoded by the OB gene is approximately 16

10 kD, which is equal to the 146 amino acid sequence carboxy- terminal to the signal
sequence. The recombinant OB protein is not modified when expressed in Pichia
pastoris. Expression of mammalian genes in Pichia generally results in the formation
of the correct protein structure [Cregg et al., Bio/Technology, 11:905-914 (1993)].
These findings suggest that the OB protein is not glycosylated and is not post-

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translationally processed in vivo. The data do not exclude the possibility that the OB protein is noncovalently bound to itself or other proteins in plasma or adipose tissue.

Although proteolytic cleavage of the protein has not been excluded, lower molecular weight forms of the OB protein were not detected by any of the antisera used, including four anti-peptide antibodies.

The OB protein has two cysteine residues and circulates as a monomer in human, and as a monomer and dimer in mouse. An intramolecular disulphide bond typical of secreted molecules is found when the human protein is expressed in *Pichia pastoris* suggesting that it is likely to be present in vivo. This is supported by the bioactivity of the recombinant bacterial protein, which has an intramolecular disulphide bond. The mouse OB protein can be found in plasma as a monomer and as a dimer. The monomer and dimer are seen when the mouse OB protein is expressed in yeast showing that the propensity of the mouse protein to form a dimer is a result of differences in the primary sequence relative to the human. While it is clear that the monomer has bioactivity, the functional activity of the dimer is unknown.

The effect of the OB protein on food intake and body weight in ob mice is dramatic. After three weeks treatment, the ob mice receiving daily injections of recombinant protein had lost 40% of their weight and were consuming 40% as much food as control animals. Moreover, the weight of the treated ob mice had not yet equilibrated at the time the experiment was terminated. The results of the pair feeding experiment indicate weight loss is a result of effects on both food intake and energy expenditure. Thus, a separate group of ob mice whose caloric intake was restricted to that of ob mice receiving protein, lost significantly less weight than the animals receiving protein. The reduction in food intake in ob/ob mice to a level lower than that of wild-type mice, within a day of receiving the OB protein, indicates that they are especially sensitive to its effects. Indeed, the OB receptor may be upregulated in these animals. Food intake of treated ob mice became relatively constant after five days of treatment. If this is the result of the protein having reached steady state levels, it would suggest that the protein has a relatively long half-life [The

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Pharmacological Basis of Therapeutics, pp. 19-45, Goodman and Gilman, eds., Pergamon Press. New York, (1990)]. This conclusion is consistent with data from parabiosis experiments [Coleman et al., 1978, supra; Weigle, Int. J. Obesity, 12:567-578 (1988)].

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Effects of recombinant protein on the body weight of wild-type mice were small but statistically significant during the first two weeks of the study. While the difference in weight between wild-type mice receiving protein vs. PBS was sustained at later time points, the statistical significance of the data greatly diminished after three 10 weeks. The early weight loss could not be accounted for by a difference in food intake. Presumably, the measurement of food intake was not precise enough to detect a decrease resulting in a one gram difference in body weight during treatment. These observations differ from the results of previous experiments in which wild-type rodents have been joined by parabiotic union to db mice, fa rats, rats with hypothalamic lesions and rats rendered obese by a high calorie diet [Coleman et al., 1978, supra; Harris et al., 1987, supra; Harris et al., "Physiological and metabolic changes in parabiotic partners of obese rats", in Hormones, Thermogenesis and Obesity, Lardy and Straatman, eds., Elsevier Science Publishing Co., New York (1989); Hervey, J. Physiol., 145:336-352 (1959)]. In each case, the wild-type animals become anorectic and lose copious amounts of weight. As the levels of OB protein are increased in db mice and fa rats and the level of OB RNA is increased in mice with hypothalamic lesions, it is likely that wild type mice can respond to OB when it circulates in plasma at a sufficiently high level. The findings reported here are consistent with the possibility that the levels of the administered protein were 25 below endogenous levels, leading to equilibration at a slightly lower body weight. Quantitation of the circulating levels of the OB protein in the treated mice will resolve this issue. While an immunoassay of the mouse protein is not yet available, immunoprecipitations have suggested that the levels of the circulating CB protein were not substantially elevated in the wild type mice receiving protein.

The lesser effect of the protein on wild type mice and the absence of a response in db mice makes it unlikely that the treatment has nonspecific or aversive effects. All of the db mice lost a small amount of weight during the treatment period, whether or not they were receiving the ob protein. The db animals were markedly hyperglycemic and the weight loss is likely to be the result of diabetes and not the experimental protocol. C57BL/Ks db/db mice often develop diabetes and begin to lose small amounts of weight when of the age of the animals used in this study (Coleman et al., 1978, supra). C57Bl/6J ob/ob mice of a similar age do not develop significant hyperglycemia. These phenotypic differences are thought to be the result of genetic differences in the strains (C57Bl/6J vs. C57Bl/Ks) carrying the mutations (Coleman et al., 1978, supra).

The failure to detect the truncated 105 amino acid protein predicted by the cDNA sequence of the OB gene in C57Bl/6J ob/ob mice suggests that the mutant protein is either degraded or not translated. However, the possibility that the antisera used do not detect this truncated protein cannot be excluded. The observed ten-fold increase in the levels of the ob protein in db mice compared to wild type suggests that the ob protein is overproduced when there is resistance to its effects. These data correlate with studies of the OB mRNA. As mentioned, previous experiments have shown that mutations of the mouse db and the rat fa genes, which map to homologous chromosomal regions, result in overproduction of a plasma factor that suppresses body weight (Truett et al., 1991, supra; Coleman, 1978, supra; Hervey, 1959, supra). In both cases, it has been suggested that the mutant animals are resistant to the effects of the OB protein. This possibility is confirmed by the observation that the OB protein has no effect on body weight or food intake when administered to db mice.

Obesity in humans could be associated with increased levels of the OB protein in

plasma in individuals who are relatively unresponsive to the hormone. On the other

hand, reduced expression of OB could also lead to obesity in which case "normal"

(i.e., inappropriately low) levels of the protein might be found. Thus, the levels of OB protein in human plasma could be a marker for different forms of obesity. In a small group of lean subjects with BMI < 25, low nanogram levels of circulating OB protein are detectable by ELISA. Significantly, variable concentrations were noted suggesting that the level of expression and/or sensitivity to the protein may play a role in determining body weight.

The site of action of the OB protein is unknown. The protein affects both food intake and energy expenditure, a finding consistent with clinical studies indicating that alterations of both systems act to regulate body weight [Leibel et al., N. Engl. J. Med., 332:621-628 (1995); Keesey et al., "Metabolic defense of the body weight set-point," in Association for Research in Nervous and Mental Disease, pp. 87-96, Stunkard and Stellar, eds., Raven Press, New York. (1984)]. The hypothalamus is likely to be downstream of OB in the pathway that controls body weight, although direct effects on a variety of organs are possible.

EXAMPLE 9: Increased Expression in Adipocytes of OB RNA in Mice with Lesions of the Hypothalamus and with Mutations at the db Locus

The gene product of the recently cloned mouse obese gene (OB) plays an important role in regulating the adipose tissue mass. OB RNA is expressed specifically by mouse adipocytes in vivo in each of several different fat cell depots including brown fat. It is also expressed in cultured 3T3-442A preadipocyte cells that have been induced to differentiate. Mice with lesions of the hypothalamus, as well as mice mutant at the db locus, express a twenty-fold higher level of OB RNA in adipose tissue. These data suggest that both the db gene and the hypothalamus are downstream of the OB gene in the pathway that regulates the adipose tissue mass and are consistent with previous experiments suggesting that the db locus encodes the OB receptor. In the db/db and lesioned mice, quantitative differences in the level of expression of OB RNA correlated with the lipid content of adipocytes. The molecules that regulate the level of expression of the OB gene in adipocytes are likely to play

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an important role in determining body weight, as are the molecules that mediate the effects of OB at its site of action.

Materials and Methods

5 In Situ Hybridization.

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White fat tissues from identical abdominal regions of wild type (wt) and db mice were processed simultaneously according to the modified method described by Richardson et al., Growth, Development & Aging, 56:149-157 (1992). Briefly, tissues were fixed in Bouin's solution for 2 hours at 4°C. They were then dehydrated by serial treatment of increasing concentrations of ethanol from 10% to 100%, each for 5 min. at 4°C. Further incubation of tissues with xylene (1hr.) and paraffin (2hr.) were performed at 65°C. Embedded wt and db/db fat tissues were sectioned and mounted by the same conditions later. Sections were baked at 65°C for 1hr. and treated with xylene and serial dilutions of ethanol from 100% to 50%, each for 3 min. at room temperature. An antisense RNA probe of OB gene was synthesized by in vitro transcription of linearized OB gene coding sequence upstream of a Sp6 RNA polymerase promoter. In situ hybridization was carried out exactly according to Schaeren-Wiemers, et al. Histochemistry, 100:431-440 (1993).

RNA Preparation and Cell Culture.

Total RNA and Northern blots were prepared as described. Stromal vascular cells and adipocytes were prepared according to Rodbell, and RNA from both fractions was prepared according to Dani et al., Mol. Cell. Endocrinol., 63:199-208 (1989); Rodbell, J. Biol. Chem. 239:375-380 (19). After sub-cloning, 3T3-F442 cells were grown in Dulbecco's modified Eagle medium containing 10% foetal bovine serum (defined as standard medium) [Dani et al., "Molecular biology techniques in the study of adipocyte differentiation", in Obesity in Europe vol 88, pp. 371-376, Bjorntorp and Rossner. Eds.. John Libbey Company Ltd., London, England (1989)]. At confluence, cells were treated in standard medium supplemented with 2 nM triiodothyronine (T3) and 17 nM insulin. Twelve days later, RNA was prepared as above.

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Gold ThioGlucose Treatment (GTG).

Two month old female CBA/J mice were treated with a single intraperitoneal injection of aurothioglucose (Sigma Catalog No. A0632) at a dose of 0.2 mg/g in normal saline. Control animals were injected with normal saline. Mice were weighed one month after the treatment. Adipose tissue RNA was isolated from those treated animals whose weight had increased more than twenty grams post- GTG treatment.

Results

The OB gene was recently found to be expressed in adipose tissue [Zhang et al., Nature, 372:425-432 (1994)]. As adipose tissue is composed of many cell types including adipocytes, preadipocytes, fibroblasts and vascular cells, in situ hybridization was performed to sections of epididymal fat pads from normal animals with sense and antisense OB riboprobes [Richardson et al., 1992, supra; Wasserman, "The concept of the fat organ" in Rodahl, Issekutz, fat as a tissue", pp. 22-92, 15 McGraw Hill, New York (1964)]. When using the antisense probe, positive signals were detectable in all of the adipocytes in the section (Figure 30 - labeled Wt). Signals were not noted when the antisense probe was hybridized to sections of brain (data not shown). Hybridization of the antisense probe to sections of adipose tissue from C57BI/Ks db/db mice was greatly increased, confirming the adipocyte specific expression of OB RNA and demonstrating a large increase in the level of OB RNA per adipocyte in these animals (Figure 30 - labeled db/db). Mice mutant at the db locus are massively obese as part of a syndrome that is phenotypically identical to that seen in C57BI/6J ob/ob mice (Bahary et al., 1990, supra).

25 OB RNA was not synthesized by adipose tissue stromal cells separated from adipocytes. As expected, cells in the adipocyte fraction expressed OB RNA using Northern blots (Figure 31). The same result was obtained using RT-PCR (data not shown). These data support the conclusion that only adipocytes express the OB gene. Data from cultured adipocytes confirm this conclusion. In these studies, 3T3-F442A cells were cultured using conditions that lead to lipid accumulation, as part of a cellular program leading to differentiation into adipocytes. OB RNA was not

expressed in exponentially growing cells, nor in confluent 3T3-F442A preadipocyte cells, which express early markers, while differentiation of these cells into adipocytes led to the expression of detectable levels of *OB* RNA (Figure 31) [Dani et al., J. Biol. Chem., 264:10119-10125 (1989)]. The level of *OB* RNA is extremely sensitive to the culture conditions, as no message was observed in late, post-confluent cells not exposed to insulin.

Hybridization studies showed that OB RNA is expressed in vivo in several different fat depots including the epididymal, parametrial, abdominal, perirenal, and inguinal 10 fat pads (Figure 32A). The precise level of expression in each of the depots was somewhat variable, with inguinal and parametrial fat expressing lower levels of OB RNA. OB RNA is also expressed in brown adipose tissue, although the level of expression is approximately 50-fold lower in brown fat relative to the other adipose tissue depots. These quantitative differences correlate loosely with previously 15 reported differences in cell size among the different fat cell depots [Johnson et al., J. Lipid Res., 13:2-11 (1972)]. The amount of OB RNA in brown fat is unaffected by cold exposure (Figure 32B). In this experiment, the level of uncoupling protein RNA (UCP) increased in brown fat after cold exposure while the level of ob RNA did not change [Jacobsson et al., J. Biol. Chem., 260:16250-16254 (1985)]. In aggregate, these data confirm that all adipocytes are capable of producing OB RNA and demonstrate a variable level of expression in different fat depots. These data support the possibility that the level of the encoded protein correlates with the total adipose tissue mass.

25 Levels of OB RNA in db/db mice and mice with lesions of the hypothalamus were measured. Lesions of the ventromedial hypothalamus (VMH) result in obesity as part of a syndrome resembling that seen in ob/ob and db/db mice [Bray et al., Metabolism, 24:99-117 (1975)]. Parabiosis experiments suggest such lesions result in over expression of a blood-borne factor that suppresses food intake and body weight (Hervey, 1959, supra). Similar results are noted when mice mutant at the db locus are parabiosed to normal mice, suggesting that the OB receptor may be encoded

by the db locus (Coleman et al., 1978, supra). Thus, obesity resulting from VMH lesions and the db mutation may be the result of resistance to the effects of the OB protein. If so, a secondary increase in the levels of OB RNA in adipose tissue would be predicted.

Hypothalamic lesions were induced in female CBA mice using the chemical gold thioglucose (GTG) [Debons et al., Fed. Proc., 36:143-147 (1977)]. This treatment results in specific hypothalamic lesions, principally in the ventromedial hypothalamus (VMH), with the subsequent development of obesity within several weeks. Usually, a single intraperitoneal injection of GTG of 0.2 mg/gm body weight results in the development of obesity within four weeks. One month old female CBA/J mice (20-25 grams) were treated with GTG and the subsequent weight gain of treated and control animals is shown (Table 2). Adipose tissue RNA was prepared from db/db mice and from those GTG treated animals that gained >20 gm. Northern blots showed a twenty-fold increase in the level of OB RNA in two month old db/db and GTG-treated mice compared to normal animals (Figure 33).

Table 2. Weight Gain in Gold Thioglucose Treated Mice

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		control $(n = 41)$	GTG (n = 93)
20	< 10 g	41, (100%)	4, (4%)
	10 g-20 g	0, (0%)	15, (16%)
	>20 g	0, (0%)	74, (80%)

Two month old female CBA/J mice were treated with gold thioglucose (GTG). Gold thioglucose (Sigma A0632) was administered intraperitonealy in normal saline solution at a dosage of 2.0 mg/g. Body weight of control and injected animals was recorded before and one month after the injection. Animals were housed five to a cage and were fed ad libitum. The amount of weight gained one month post-injection is shown in the Table 2. Animals with a body weight gain greater that 20 g one month after injection were selected for further study.

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Discussion

The gene product of the mouse OB gene circulates in mouse and human plasma where it may act to regulate the adipose tissue mass. Further studies on the regulation of expression and mechanism of action of OB will have important implications for our understanding of the physiologic pathway that regulates body weight.

The present Example shows that the OB gene product is expressed exclusively by adipocytes in all adipose tissue depots. This result is consistent with the possibility that the protein product of the OB gene correlates with the body's lipid stores. Moreover OB RNA is upregulated twenty fold in db mice and mice with hypothalamic lesions. In these animals, the actual increase in the level of OB RNA per cell is likely to be even higher than twenty-fold since the adipocyte cell size is increased approximately five-fold in these animals (see Figure 30) (Debons et al., 1977, supra). These data position the db gene and the hypothalamus downstream of 15 OB in the pathway that controls body weight and is consistent with the hypothesis that the OB receptor is encoded at the db locus [Coleman et al., Diabetologia 14:141-148 (1978)]. The molecular cloning of the OB receptor and/or the db gene will resolve this issue. The increase in the level of OB RNA in db/db and GTG-treated mice also suggests a non cell-autonomous function of the OB gene product in fat cells [Ashwell 20 et al., Proc. R. Soc. Lond., 195:343-353 (1977); Ashwell et al., Diabetologia, 15:465-470]. Thus, if the encoded protein acted directly on fat cells to inhibit growth or differentiation, the overexpression of the wild type OB gene in GTG treated mice would result in a lean phenotype.

25 The most parsimonious explanation of these data is that the OB protein functions as an endocrine signaling molecule that is secreted by adipocytes and acts, directly or indirectly, on the hypothalamus. Direct effects on the hypothalamus would require that mechanisms exist to allow passage of the OB gene product across the blood brain barrier. Mechanisms involving the circumventricular organ and/or specific transporters could permit brain access of a molecule the size of that encoded by the OB gene [Johnson et al., FASEB J., 7:678-686 (1983); Baura et al., J. Clin. Invest.,

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92:1824-1830 (1993); Pardridge, Endocrine Reviews, 7:314-330 (1986)]. However, this hypothesis must be considered with caution until the means by which the protein might cross the blood brain barrier have been identified. Moreover, possible effects on other target organs will need to be evaluated.

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The fat cell signal(s) that are responsible for the quantitative variation in the expression level of the OB gene is not vet known but correlates with differences in adipocyte cell size. Adipocytes from db/db mice are five times as large as those from normal mice, with a cell size of approximately 1.0 µg lipid/cell (Johnson et al., 1972, 10 supra). Prior evidence has indicated that fat cell lipid content and/or size is an important parameter in determining body weight [Faust et al., Am. J. Physiol., 235:279-286 (1978); Faust et al., Science, 197:393-396 (1977)]. It could be that each fat cell expresses a low level of OB RNA that further increases in proportion to the cell size. It is also possible that cell size is not the sensed parameter, but merely 15 correlates with the intracellular signal that increases the expression of the OB gene in adipocytes from db/db and VMH- lesioned mice. In any case, the components of the signal transduction pathway regulating the synthesis of OB RNA are likely to be important in determining body weight. Genetic and environmental influences that reduce the level of expression of OB would act to increase body weight, as would influences that decreased sensitivity to the encoded protein. The specific molecules that regulate the level of expression of the OB gene are as yet unknown, and await a determination of the level(s) of gene control that leads to quantitative variation in the level of OB RNA, and an examination of the regulatory elements of the OB gene. The identification of the molecules that regulate the expression of the OB gene in adipocytes, and those that mediate the effects of the encoded protein at its site(s) of action, will greatly enhance our understanding of the physiologic mechanisms that regulate body weight.

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EXAMPLE 10: RNA Expression Pattern and Mapping on the Physical, Cytogenetic, and Genetic Maps of Chromosome 7

OB RNA is expressed at high levels in human adipose tissue, and at substantially lower levels in placenta and heart. The human OB gene maps to a large yeast artificial chromosome (YAC) contig derived from chromosome 7q31.3. In addition to confirming the relative location of the gene based on mouse-human comparative mapping, this study has identified 8 established microsatellite markers in close physical proximity to the human OB gene. Since mutations in mouse OB can result in a syndrome that closely resembles morbid obesity in humans, these genetic markers represent important tools for studying the possible role of the OB gene in inherited forms of human obesity.

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Materials and Methods

Northern Blot Analysis.

Total RNA was prepared from adipose tissue using the method of Chirgwin et al., Biochem., 18:5294-5299 (1979). Northern blots, radiolabeling, and hybridizations were performed as described (Zhang et al., 1994, supra). Northern blots of polyA* RNA (human MTN, human MTN II, and human fetal MTN II) were obtained from CLONETECH (Palo Alto, CA), as were PCR primers used to generate the radiolabeled human actin probe.

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STS Development.

Sequence tagged-site (STS)-specific PCR assays were developed and optimized essentially as described [(Green et al., PCR Methods Applic., 1991; Green et al., Genomics, 11.548-564 (1991), Green, "Physical mapping of human chromosomes: generation of chromosome-specific sequence-tagged sites", in Methods in Molecular Genetics Vol. 1, Gene and Chromosome Analysis (Part A), pp. 192-210, Adolph ed., Academic Press, Inc., San Diego (1993); Green et al., Hum. Mol. Genet.,

3:489-501 (1994)]. Each STS is named using the prefix 'sWSS' followed by a unique number. Details about the 19 STSs reported here are provided in Table 3, with additional information (e.g., PCR reaction conditions, complete DNA sequence) available in GenBank and/or the Genome Data Base (GDB). For the microsatellite-specific STSs, the oligonucleotide primers used in the PCR assays (Table 3) corresponded either to those employed for genotype analysis (Table 4), or those designed (most often with the computer program OSP) [Hillier et al., PCR Methods Applic., 1:124-128 (1991)] using the DNA sequence available in GenBank. Table 3 illustrates STSs in the YAC contig containing the human OB gene

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	GDB ID No.	G00-334-404	G00-455-262	G00-455-247	G00-455-244	G00-307-733	G00-344 044	G00-334-839	G00-455-238	G00-307-700	G00-455:241
SEQ	0 N 6 9	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	£ 4	₹ 4	47	50	51	25 25	\$ \$	57 88	\$ 8
	Size 72 CT	112	490	142	112	154	137	501	981	144	200
Table 3	PCR Pimess Caagacaaatgagataagg Agagttacagctttacag	CTAAACACCTITCCATTCC TTATATTCACTTTTCCCCTCTC	TGCAGTAAGCTGTGATTGAG GTGCAGCTTTAATTGTGAGC	AGTGTTGTGTTTCTCCTG AAAGGGGATGTGATAAGTG	GGTGTTACGTTTAGTTAC GGAATAATGAGAAGATTG	GCTCAACTGACAGAAAAC GACTATGTAAAAGAAATGCC	AAAGGGCTTCTAATCTAC CCTTCCAACTTCTTTGAC	TAAACCCCTTTCTGTTC TTGCATAATAGTCACACCC	CCAAAATCAGAATTGTCAGAAG 186 AAACCGAAGTTCAGATACAG	AATATCTGACATTGGCAC TTAGACCTGAGAAAAGAG	GTTGCACAATACAAAATCC CTTCCATTAGTGTCTTATAG
	Source YAC End	Lambda Clone	Genetic Marker	Genetic Marker	Genetic Marker	Genetic Marker	YAC End	YAC Intert	YAC End	Genetic Marker	YAC End
	Locus D7S2185	D752016	D751498	D751873	D7S1874	D7\$680	D7S1916	D7S1674	D7S2186	D7S514	D7S2184
	Alize		UT528	AFM#065zg9	AFM±125wh1	AFM309yfl				AFM218xf10	
	STS Name aWSS1734	«WSS494	#W55883	•WSS2359	,WSS2336	•WSS1218	*WSS1402	8WSS999	187128WA	8WSS1174	•WSS2061

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	GDB ID No.	G00.455-253	G00-455-259	G00-307-815	G00-307-652	G00-334-119	G00-455-256	G00-334-251	G00-455-250
SEQ	_0.0N	19	2.2	2.8	68	69 02	1, 2,	17	75 76
	Size (bp)	117	3GA 153	2 7	8	116	<u>8</u>	123	
1000	PCR Primers	ATCACTACACACCTAATC CCATTCTACATTTCCACC	GGCTGTGTGAGCAAGATCCTAGGA TTGCCAGGCAAAGAGGGCTGGAC	CTCAGGTATGTCTTTATC TGTCTCTGCATTCTTTTC	GACACATACAAACACAAG ATTGAGTTGAGTGTAGTAG	CAGGGATTTCTAATTGTC AAAAGATGGAGGCTTTTG	CGTTAAGGGAAGGAACTCTGG TGGCTTAGAGGAGTCAGGGA	ACCAGGGTCAATACAAAG TAATGTGTCCTTCTTGCC	CAATCCTGGCTTCATTTG AAGGTGGGTAGGATGCTA
	Source	YAC End	Gene	Genetic Marker	Genetic Marker	YAC End	Gene	Lambda Clone	Genetic Marker
	Locus	D7S2187	PAX4	D7S635	D7S504	D7S1943	08	D7S1956	D781875
	Aline		Pax-4	AFM206xc1	AFM199xh12		80		AFMa345wc9
	STS Name	*WSS2588	*WSS808	«WSS1392	#WSS1148	665188Wa	«WSS2619	*WSS404	•WSS2367

The 19 chromosome 7-specific STSs mapped to the YAC contig containing the human OB gene (Figure 35) are listed. In each case, the designated 'sWSS' name, relevant alias, GDB-assigned locus name, STS source, PCR primer sequences, STS size, and GDB identification number are indicated. The sources of STSs are as follows: 'YAC End' (isolated insert end of a YAC) (Green, 1993, supra), 'Lambda Clone' (random chromosome 7-specific lambda clone) (Green et al. 1991, supra; Green, 1993, supra). 'Genetic Marker' (microsatellite marker, see Table 2) (Green et al. 1994, supra), 'YAC Insert' (random segment from YAC insert), and 'Gene' (gene-specific STS). Note that for some genetic marker-specific STSs, the PCR primers used for identifying YACs (listed in this table) are different from those used for performing genotype analysis (Table 4), since the detection of YACs containing a genetic marker does not require amplification of the polymorphic tract itself. All of the indicated PCR assays utilized an annealing temperature of 55°C, except for sWSS494, sWSS883, sWSS1529, and sWSS2619 (which used 50°C), sWSS999 and sWSS1174 (which used 60°C), and sWSS808 (which used 65°C). Additional details regarding the STS-specific PCR assays are available in GDB.

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able 4

SEQ

Microsatellile markers in the YAC contig containing the human ob gene.

:				<u>e</u>	i di
Marker Name	Type	Cocus	Primera	N	COD ID NO.
UT528	Tetra.	D751498	TGCAGTAAGCTGTGATTGAG GTGCAGCTTTAATTGTGAGC	7.7 7.8	G00-312-446
AFMa065zg9	(CA),	D7S1873	AGCTTCAAGACTTTNAGCCT GGTCAGCAGCACTGTGATT	97	G00-437-253
AFMa125wh1	(CA)	D7S1874	TCACCTTGAGATTCCATCC AACACCGTGGTCTTATCAAA	81	G00-437-263
AFM309yf10	(CA).	D75680	CATCCAAGTTGGCAGTTTTT AGATGCTGAATTCCCAGACA	25.25	G00-200-283
AFM218xf10	(V)	D7S514	TGGCCACCAGCAAA TGCAGTTAGTGCCAATGTCA	88 88	G00-188-404
AFM206xc1	(CA),	D78635	CCAGGCCATGTGGAAC AGTTCTTGGCTTGCGTCAGT	88	G00-199-240
AFM199xh12	(CA),	D7S504	TCTGATTGCTGGCTGC GCGCGTGTGTATGTGAG	68	G00-188-280
AFMa345wc9	(CA),	D7S1875	AGCTCTTGGCAAACTCACAT GCCTAAGGGAATGAGACACA	91	G00-437-259

The eight microsatellite markers mapped to the YAC contig containing the human OB gene (Figure 35) are listed. In each case, the marker name (indicated as the alias in Table 3), type of microsatellite motif (tetranucleotide- 'Tetra' repeat or (CA), repeat), GDB-assigned locus name, primer sequences utilized for PCR-based genotype analysis, and GDB identification number are indicated. Additional details regarding the PCR assays and the polymorphisms are available in GDB.

The human OB-specific STS (sWSS2619) was designed using DNA sequence obtained from the 3' untranslated region of the cDNA. The human Pax4-specific STS 10 (sWSS808) was developed using the following strategy. Oligonucleotide primers specific for the mouse Pax4 gene (GGCTGTGTGAGCAAGATCCTAGGA) (SEQ ID NO:63) and (GGGAGCCTTGTCCTGGGTACAAAG) (SEQ ID NO:93) [Walther et al., 1991, Genomics 11:424-434) (1991)] were used to amplify a 204-bp fragment from human genomic DNA (which was the same size product as that generated from This PCR assay was not suitable for identifying 15 mouse genomic DNA). corresponding YACs, since a similarly-sized (200-bp) product was also amplified from yeast DNA. However, DNA sequence analysis of the PCR product generated from human DNA revealed substitutions at 20 positions among the 156 bases analyzed Using this human-specific sequence, a new primer (data not shown). 20 (TTGCCAGGCAAAGAGGGCTGGAC) (SEO ID NO:64) was designed and used with the first of the above mouse Pax4-specific primers (see Table 3). The resulting human Pax4-specific PCR assay did not amplify a significant product from yeast DNA and was thus used for identifying corresponding YACs.

Identification of YACs by PCR-based screening.

25

Most of the YACs depicted in Figure 35 were derived from a collection of clones highly enriched for human chromosome 7 DNA (the 'chromosome 7 YAC resource') (Green et al., 1995. supra) using a PCR-based screening strategy [Green et al., 1995. supra; Greena et al., Proc. Natl. Acad. Sci. USA, 87:1213-1217 (1990)]. In a few cases, clones were isolated by PCR-based screening (Greena et al., 1990, supra) of available total human genomic YAC libraries constructed at CEPH [Dausset et al.,

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Behring Inst. Mitt., 91:13-20 (1992); Albertsen et al., Proc. Natl. Acad. Sci. USA, 87:4256-4260 (1990)] or ICI [Anand et al., Nucl. Acids Res., 17:3425-3433 (1989); Anand et al., Nucl. Acids Res. 18:1951-1956 (1990)]. Each YAC is named using the prefix 'yWSS' followed by a unique number.

Results and Discussion

5

Examination of the tissue expression of the human OB gene by Northern blot analysis revealed that OB RNA is expressed at a high level in human adipose tissue and much lower levels in placenta and heart (Figure 34). The size of the RNA (approximately 10 4.5 kb) was equivalent in human and mouse as well as in each of the expressing tissues. In these studies, five-fold higher signals were seen in 10 µg of total adipose tissue RNA, as in 2 µg of polyA+ placental RNA. A five-fold lower signal was seen in polyA+ RNA from heart compared to placenta. It is estimated that the level of OB RNA is approximately 250-fold lower in placenta than in adipose tissue. In this 15 experiment, OB RNA was not detected in any of the other tissues analyzed, including brain, lung, liver, skeletal muscle, kidney, and pancreas. Additional experiments did not reveal OB RNA in spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, or in fetal brain, liver, or kidneys (data not shown). It is possible that OB is expressed at an undetectable level (by Northern blot analysis) 20 in these latter tissues or in other tissues that were not studied. The observed pattern of expression in human differs somewhat from mouse, in which OB RNA is detected almost exclusively in adipose tissue.

Comparative mapping of the OB gene region in the mouse and human genomes. The mouse OB gene is located on proximal chromosome 6 in a region homologous with a portion of human chromosome 7q. Genes within this segment include (from proximal to distal): the Met protooncogene, the cystic fibrosis transmembrane conductance regulator (Cftr), paired box-containing gene 4 (Pax4), OB, and carboxypeptidase A (Cpa) (Zhang et al., 1994, supra; Friedman et al., 1991, supra).

30 In the mouse, genetic mapping was used to demonstrate that Pax4 is tightly linked to ob [Walther et al., 1991, supra; Zhang et al., 1994, supra]. The physical distance

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between OB and Pax4 was found to be approximately one megabase pairs (Mb) (Zhang et al. 1994, supra). Based on these comparative mapping studies, it was expected that the human OB gene would reside between Pax4 and CPA on chromosome 7q. Furthermore, since human CFTR [Heng et al., Cell Genet., 62:108-109 (1993)] and Pax4 [Tamura et al., Cytogenet. Cell Genet., 66:132-134 (1994)] were mapped by fluorescence in situ hybridization (FISH) to 7q31.3 and 7q32, respectively, the most likely cytogenetic position of the human OB gene would be in the vicinity of the 7q31.3-q32 boundary.

Mapping the OB gene on human chromosome 7.

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An STS (\$WSS2619) amplifying a small segment of the 3' untranslated region of the human OB gene was used to screen a collection of YAC clones that is highly enriched for human chromosome 7 DNA (Green et al., 1995a, Genomics 25: 170-183), and 9 YACs were identified (yWSS691, yWSS1332, yWSS1998, yWSS2087, yWSS3319, yWSS3512, yWSS4975, yWSS4970, and yWSS5004). To verify that these YACs contain the authentic human OB gene, two additional experiments were performed. First, each of the YACs was tested with a second human OB-specific PCR assay, and all were found to be positive (data not shown). Second, yeast DNA from each clone was digested with EcoRI and analyzed by gel-transfer hybridization, using a human OB cDNA-derived probe. In all instances, a single hybridizing band was seen; and this band was the same size in the YACs and a PI clone known to contain the human OB gene (data not shown).

Using the computer program SEGMAP (Green and Green, 1991, supra) and other

YAC-based STS-content data that we have generated for chromosome 7 (Green et al. 1991, supra; Green et al. 1994, supra; Green et al. 1995, supra), the human OB gene was found to reside within the YAC contig depicted in Figure 35. Specifically, this contig consists of 43 overlapping YACs and 19 uniquely-ordered STSs. Details about each of the 19 STSs are provided in Table 3. In addition to the OB-specific STS, the contig also contains an STS (sWSS808) specific for the human Pax4 gene (Tamura et al. 1994, supra; Stapleton et al., 1993, Nature Genet, 3:292-298), 7 STSs

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derived from chromosome 7-specific YACs, 2 STSs derived from chromosome 7-specific lambda clones, and, importantly, 8 microsatellite-specific STSs. Additional details about these 8 genetic markers, including sequences of the primers used for genotype analysis, are provided in Table 2. Of note, there is redundant YAC-based connectivity throughout the contig (i.e., there are 2 or more YACs connecting each adjacent pair of STSs), lending strong support for the relative order of STSs shown in Figure 35.

As depicted in Figure 35, the predicted orientation of the human OB-containing YAC contig is such that sWSS1734 is the centromeric-most STS (i.e., closest to CFTR) whereas sWSS2367 is the telomeric-most STS (i.e., closest to CPA). This orientation is predominantly based on comparative mapping data, which places Pax4 proximal and OB distal within the syntenic block present in mouse and human DNA (Zhang et al. 1994, supra). The OB gene maps near the telomeric end of the contig, based on the placement of the OB-specific STS (sWSS2619).

While the contig shown in Figure 35 was deduced by SEGMAP without consideration of YAC sizes (thereby displaying STSs equidistant from one another), a similar analysis of the data by SEGMAP that accounted for YAC sizes indicated that the total size of the region covered by the contig is just over 2 Mb (data not shown). Thus, while all 8 of the microsatellite-specific STSs (Table 4) are contained within a genomic interval spanning roughly 2 Mb, the 3 closest to the telomeric end of the contig (sWSS1392, sWSS1148, and sWSS2367) are particularly close to the OB gene itself (perhaps within an interval as small as approximately 500 kb). In fact, all 3 of the latter STSs are present in at least 1 of the human OB-containing YACs. Of note, the interval between human Pax4 (sWSS808) and ob (sWSS2619) is estimated to be approximately 400 kb, whereas this region was predicted to span approximately 1 Mb in mouse (Zhang et al., 1994, supra). Finally, 3 of the YACs within the contig (yWSS691, yWSS999, and yWSS2935) have also been analyzed by FISH, and each was found to hybridize exclusively to 7q31.3. One of these YACs (yWSS691) contains the OB-specific STS, while the other 2 clones contain the Pax4-specific STS.

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The latter results are generally consistent with the previous cytogenetic assignment of human Pax4 to 7q32 (Tamura et al. 1994, supra). Based on these data, the human OB gene can be assigned to cytogenetic band 7q31.3.

5 EXAMPLE 11: Human OB Polypeptide is Biologically Active in Mice

Groups of 10 ob/ob mice were treated by i.p. injection with $10 \mu g/g/\text{day}$ recombinant (bacterial) human and murine OB polypeptide or saline. After four days, the group receiving saline gained 0.3 g. The group receiving murine OB lost 3.2 g. The group receiving human OB lost 2 g (p < .01 compared to saline controls). These groups were also tested for food intake. The data for food intake are shown in Table 5; the data for body mass are shown in Table 6.

Table 5
Food intake/day (g) of treated ob/ob mice (value ± S.Dev)

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
saline	13.4 ± 2.6	12.8	12.8	13.1	14.0	12.3	12.4	8.3
murine OB	14.9	3.7	4.4	5.1	8.9	8.1	8.7	3.5
human OB	14.3	10.3	8.7	7.0	8.9	5.3	3.8	13.0

Table 6 Body weight and weight change in treated ob/ob mice (value \pm S.Dev)

Treatment	Body Weight (Day 0)	Body Weight (Day 4)	Percent change (Day 0 to 4)	Body Weight (Day 6)	Percent Change (Day 0 to 6)
saline	39.9 ± 1.8	40.7 ± 1.6	0.8 ± 0.5	41.1 ± 2.2	1.2 ± 1.1
murine OB	39.5 ± 2.1	36.2 ± 2.0	-3.3 ± 1.2	36.3 ± 2.2	-3.1 ± 1.2
human OB	39.5 ± 2.0	37.6 ± 1.7	-2.0 ± 1.0	36.1 ± 1.3	-3.5 ± 1.3

These data demonstrate that human OB is biologically active in mice.

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Wild-type mice (C57Bl6J +/?) were treated with 10 μ g/g/day i.p. of recombinant murine OB, and body mass was measured every four days. The results are shown in Table 7.

Table 7

Body mass (g) of normal mice receiving OB

Body	mass (g) of	HOTHIAI III	CC TOCCT		
Treatment	Day 0	Day 4	Day 8	Day 12	Day 16
	22.6±1.4	22.2 ± 1.2	22.5±1.3	23	22.5
saline	22.4±1.5				21.8
murine OB	22.411.5	20.01			

These data demonstrate that OB affects the body mass of wild-type as well as obese (ob/ob) mice, albeit to a much smaller degree.

EXAMPLE 13: OB Polypeptide Administered By Continuous Pump Infusion

This example demonstrates that continuous infusion of OB polypeptide results in weight loss in normal mice. Normal (non-obese) mice were administered murine ob polypeptide via osmotic pump infusion. A dosage of 0.5 mg protein/kg body weight/day resulted in a 4.62% loss (+/- 1.34%) from baseline weight by the 6th day of infusion.

Materials and Methods

Animals

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Wild type (+/+) C57B16 mice were used in this Example. Mice were single-housed, and maintained under humane conditions. The age of the mice at the initial time point was 8 weeks, and the animals were weight stabilized. Ten mice were used for each cohort (vehicle vs. protein).

Feeding and weight measurement

Mice were given ground rodent chow (PMI Feeds, Inc.) in powdered food feeders (Allentown Caging and Equipment), which allowed a more accurate and sensitive measurement of food intake than use of regular block chow. Weight was measured

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at the same time each day (2:00 p.m.), for a period of 6 days. Body weight on the day prior to infusion was defined as baseline weight.

Cloning of Murine OB DNA

5 The cloning of the murine OB DNA for expression in E. coli was performed as follows. The DNA sequence as deduced from the published peptide sequence that appeared in (Zhang et al., 1994, supra, i.e, Example 1, supra) was reverse translated using E. coli optimal codons. The terminal cloning sites were Xbal to BamHI. A ribosomal binding enhancer and a strong ribosomal binding site were included in front of the coding region. The duplex DNA sequence was synthesized using standard techniques. Correct clones were confirmed by demonstrating expression of the recombinant protein and presence of the correct OB DNA sequence in the resident plasmid. The amino acid sequence (and DNA sequence) is as follows:

Recombinant murine met OB (double stranded) DNA and amino acid sequence. (Seq. ID, NOS:94 and 95):

20	9	AGI																			TCA	
															M	v	P	I	Q	K	v	-
25	69	-+-			+				+			-+-			+				+		TCA AGT	128
30		Q	D	D	T	ĸ	T	L	I	K	T	I	v	T	R	I	N	D	I	s	H	-
,,	129	-+-							+			-+-			+				+			188
35		T	Q	s	v	s	A	K	Q	R	v	T	G	L	D	F	I	P	G	L	н	-
10	189	-+-			+			GTI	+			- + -							÷			248
•0		P	I	L	s	L	s	ĸ	M	D	Q	T	L	A	v	Y	Q	Q	v	L	T	-
	249	-+-			+			CGI	+						+				+			308

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SLPSQNVLOIANDL GCTGCACCTGCTGGCATTCTCCAAATCCTGCTCCCTGCCGCAGACCTCAGGTCTTCAGAA 5 CGACGTGGACGACCGTAAGAGGTTTAGGACGAGGGACGGCGTCTGGAGTCCAGAAGTCTT LHLLAFSKSCSLPQT ACCGGAATCCCTGGACGGGGTCCTGGAAGCATCCCTGTACAGCACCGAAGTTGTTGCTCT 10 TGGCCTTAGGGACCTGCCCCAGGACCTTCGTAGGGACATGTCGTGGCTTCAACAACGAGA PESLDGVLEASLY 15 GTCCCGTCTGCAGGGTTCCCTTCAGGACATCCTTCAGCAGCTGGACGTTTCTCCGGAATG CAGGGCAGACGTCCCAAGGGAAGTCCTGTAGGAAGTCGTCGACCTGCAAAGAGGCCTTAC SRLOGSLODILOOL 20 TTAATGGATCC 499 AATTACCTAGG 25

The plasmid expression vector used to produce the protein was pCFM1656, American Type Culture Collection (ATCC) Accession No. 69576. The above DNA was ligated into the expression vector pCFM1656, which had been linearized with XbaI and BamHI, and transformed into the E. coli host strain, FM5. E. coli FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from E. coli K-12 strain [Bachmann et al., Bacteriol. Rev., 40:116-167 (1976)] and contain the integrated lambda phage repressor gene, clust [Sussman et al., C.R. Acad. Sci., 254:1517-1579 (1962)]. Vector production, cell transformation, and colony selection were performed by standard methods. e.g., Sambrook et al., 1989, supra. Host cells were grown in LB media

Expression Vector and Host Strain.

Administration of Protein or Vehicle

Recombinant murine OB polypeptide was used for the present experiments, generally
at a concentration of about 0.9 mg/ml phosphate buffered saline, pH 7.4. The amino
acid sequence (and DNA sequence) used is set out immediately above. Protein or
vehicle (phosphate buffered saline, pH 7.4) were administered by osmotic pump
infusion. Alzet osmotic minipumps (Alza, Palo Alto, CA, model no. 1007D) were
surgically placed in each mice in a subcutaneous pocket in the subscapular area. The

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pumps were calibrated to administer 0.5 ml protein in solution per hour for a dosage of 0.5 mg protein/kg body weight/day. Control animals were infused with phosphate buffered saline (pH 7.4) via an Alzet osmotic minipump.

- 5 Fermentation Process. A three-phase fermentation protocol known as a fed-batch process was used to prepare the protein. Media compositions are set forth below.
- Batch. A nitrogen and phosphate source were sterilized (by raising the temperature to 122°C for 35 minutes, 18-20 psi) in the fermentation vessel (Biolafitte, 12 liter capacity). Upon cooling, carbon, magnesium, vitamins, and trace metal sources were added aseptically. An overnight culture (16 hours or more) of the above recombinant murine protein-producing bacteria of 500 ml (grown in LB broth) was added to the fermentor.
- 15 Feed I. Upon reaching between 4.0-6.0 O.D.₆₀₀, Feed I was added to cultures. The glucose was added at a limiting rate in order to control the growth rate (μ). An automated system (called the Distributive Control System) was programmed to control the growth rate at 0.15 generations hr⁻¹.
- 20 Feed II. When the O.D. reached 30, the temperature was slowly increased to 42°C and the feed was changed to Feed II, described below. The fermentation was then allowed to continue for 10 hours with sampling every 2 hours. After 10 hours, the contents of the fermentor were chilled to below 20°C and harvested by centrifugation.

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Media Composition:

	Batch:	10 g/L	Yeast extract
		5.25 g/L	$(NH_4)_2SO_4$
5		3.5 g/L	K ₂ HPO ₄
		4.0 g/L	KH₂PO₄
		5.0 g/L	Glucose
		1.0 g/L	MgSO ₄ •7H ₂ O
		2.0 ml/L	Vitamin Solution
10		2.0 ml/L	Trace Metal Solution
		1.0 ml/L	P2000 Antifoam
	Feed I:	50 g/L	Bacto-tryptone
		50 g/L	Yeast extract
		450 g/L	Glucose
15		8.75 g/L	MgSO ₄ •7H ₂ O
		10 ml/L	Vitamin Solution
		10 ml/L	Trace Metal Solution
	Feed II:	200 g/L	Bacto-tryptone
		100 g/L	Yeast extract
20		110 g/L	Glucose

Vitamin Solution (Batch, Feed I): 0.5 g biotin, 0.4 g folic acid, and 4.2 g riboflavin, were dissolved in 450 ml H₂O and 3 ml 10 N NaOH, and brought to 500 ml with H₂O. Fourteen grams of pyridoxine-HCl and 61 grams of niacin were dissolved 150 25 ml H₂O and 50 ml 10 N NaOH, and brought to 250 ml with H₂O. Fifty-four grams of pantothenic acid were dissolved in 200 ml H₂O, and brought to 250 ml. The three solutions were combined and brought to 10 liters total volume.

Trace Metal Solution (Batch, Feed I): 30 Ferric Chloride (FeCl₃·6H₂O): 27 g/L Zinc Chloride (ZnCl₂4H₂O): 2 g/L

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Cobalt Chloride (CoCl₂ 6H₂O): 2 g/L

Sodium Molybdate (NaMoO, 2H,O): 2 g/L

Calcium Chloride (CaCl₂·2H₂O): 1 g/L

Cupric Sulfate (CuSO₄:5H₂O): 1.9 g/L

5 Boric Acid (H₃BO₃): 0.5 g/L

Manganese Chloride (MnCl $_2$ 4H $_2$ O): 1.6 g/L

Sodium Citrate dihydrate: 73.5 g/L

Purification Process for Murine OB polypeptide

- 10 Purification was accomplished by the following steps (unless otherwise noted, the following steps were performed at 4°C):
- Cell paste. E. coli cell paste was suspended in 5 times volume of 7 mM of EDTA, pH 7.0. The cells in the EDTA were further broken by two passes through
 a microfluidizer. The broken cells were centrifuged at 4.2k.rpm for 1 hour in a Beckman JB-6 centrifuge with a J5-4.2 rotor.
 - Inclusion body wash #1. The supernatant from above was removed, and the pellet was resuspended with 5 times volume of 7 mM EDTA, pH 7.0, and homogenized. This mixture was centrifuged as in step 1.
- 20 3. Inclusion body wash #2. The supernatant from above was removed, and the pellet was resuspended in ten times volume of 20 mM Tris, pH 8.5, 10 mM DTT, and 1% deoxycholate, and homogenized. This mixture was centrifuged as in step 1.
- Inclusion body wash #3. The supernatant from above was removed and the
 pellet was resuspended in ten times volume of distilled water, and homogenized. This
 mixture was centrifuged as in step 1.
 - 5. Refolding. The pellet was refolded with 15 volumes of 10 mM HEPES, pH 8.5, 1% sodium sarcosine (N-lauryl sarcosine), at room temperature. After 60 minutes, the solution was made to be 60 mM copper sulfate, and then stirred overnight.
- 30 6. Removal of sarcosine. The refolding mixture was diluted with 5 volumes of 10 mM Tris buffer, pH 7.5, and centrifuged as in step 1. The supernatant was

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collected, and mixed with agitation for one hour with Dowex 1-X4 resin, 20-50 mesh, chloride form (at 0.066% total volume of diluted refolding mix). This mixture was poured into a column and the cluant was collected. Removal of sarcosine was ascertained by HPLC.

- 7. Acid precipitation. The cluant from the previous step was collected, and the pH adjusted to pH 5.5, and incubated for 30 minutes at room temperature. This mixture was centrifuged as in step 1.
- Cation exchange chromatography. The pH of the supernatant from the previous step was adjusted to pH 4.2, and loaded on CM Sepharose Fast Flow.
 Twenty column volumes of salt gradient were used at 20 mM NaOAC, pH 4.2, 0 M to 1.0 M NaCl.
- HIC chromatography. The CM Sepharose pool of peak fractions (ascertained from ultraviolet analysis) from the above step was adjusted to be 0.2 M ammonium sulfate. A 20 column volume reverse salt gradient was done at 5 mM NaOAc, pH
 4.2, with 0.4 M to 0 M ammonium sulfate. This material was concentrated and disfiltered into PBS.

Results

Presented below are the percent (%) differences from baseline weight in C57Bl6J

omice (8wks old):

Table 8
Weight Loss Upon Continuous Infusion

Time (days)	Vehicle (PBS)	Recombinant OB polypeptide
Days 1-2	3.24 +/- 1.13	1.68 +/- 1.4
Days 3-4	4.3 +/97	-2.12 +/79
Days 5-6	4.64 +/96	-4.62 +/- 1.3

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As can be seen, at the end of a 6 day continuous infusion regime, animals receiving the OB polypeptide lost over 4% of their body weight, as compared to baseline. This is a substantially more rapid weight loss than has been observed with intraperitoneal (i.p.) injection. Weight loss of only 2.6-3.0% was seen at the end of a 32-day injection period, in wild-type (normal) mice, with daily i.p. injections of recombinant murine OB polypeptide at a 10 mg/kg dose, and had not been more than 4% at any time during the dosing schedule (data not shown). The present data indicate that with continuous infusion, a 20-fold lower dosage (0.5 mg/kg vs. 10 mg/kg) achieves more weight loss in a shorter time period.

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The results seen here are statistically significant, e.g., -4.62% with p < .0001.

EXAMPLE 14: Cloning and Expression of a Recombinant Human OB Polypeptide Analog

This example provides compositions and methods for preparation of an analog recombinant human version of the OB polypeptide.

20 The human version of OB DNA was constructed from the murine OB DNA, as in Example 13, above, by replacing the region between the Mlul and BamHI sites with duplex DNA (made from synthetic oligonucleotides) in which 20 codon substitutions had been designed. Codons for arginine at mouse mature position 35, and leucine at mouse mature position 74, were unchanged. The Mlul site is shown under the solid line in the sequence below. This DNA was put into the pCFM 1656 vector (ATCC Accession No. 69576), in the same fashion as the recombinant murine protein, as described above.

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Recombinant human met OB (Double-Stranded) DNA and amino acid sequence (Seq. ID. NOS:96 and 97)

5	1	GT	ATA	CCA'	TGG	CTA	GGT	CII	TCA	AGT	CCT	GCT	GTG	GTT	TTG	GAA	TTA	ATT	TTG	CTA	GCAA	60
			M	v	P	I	Q	ĸ	v	Q	D	D	T	ĸ	T	L	1	K	T	I	v	-
10	61				- 4 -			+				+			-+-			+			AGGC TCCG	120
15		T	R	1	N	D	I	s	H	Ť	Q	s	v	s	s	ĸ	Q	R	v	T	G	-
15	121							+				+			-+-			+			GGAC	180
20																					L	
25	181											4			-+-			+			TAAC	240
23		A	v	Y	Q	Q	I	L	T	s	Ħ	P	s	R	N	v	L	Q	I	S	N	-
30	241				-+-			+				+			-+-			+			GGAC	300
		D	L	E	N	L	R	D	L	L	н	v	L	A	F	s	ĸ	s	С	H	L	-
35	301				- + -			+				+			-+-			+			CGGT	360
		P	w	A	s	G	L	E	T	L	D	s	L	G	G	v	L	E	A	s	G	-
40	361				- + -			+				+			-+-			+			TTGG + AACC	420
45		Y	s	T	E	v	v	A	L	s	R	L	Q	G	s	L	Q	D	M	L	w	-
45	421							GGG						454								
								ccc														
50		Q	L	D	L	s	P	G	С	٠												

Fermentation

55 Fermentation of the above host cells to produce recombinant human OB
polypeptide was accomplished using the conditions and compositions as described
above for recombinant murine material. The results were analyzed for yield

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(grams/liter), pre-purification, of the recombinant human OB material (and minor amounts of bacterial protein), and correlated to analyze bacterial expression:

Table 9

Analysis of Human OB Polypeptide Expression

	Timepoint	OD	Yield	Expression
		(@600 nm)	(g/L)	(mg/OD·L)
	Ind. + 2 hrs.	47	1.91	41
10	Ind. + 4 hrs.	79	9.48	120
	Ind. + 6 hrs.	95	13.01	137
15	Ind. + 8 hrs.	94	13.24	141
	lnd. + 10 hrs.	98	14.65	149

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abbreviations: Ind. + _ hours means the hours after induction of protein expression, as described in Example 13 for the recombinant murine material using pCFM 1656

O.D.: optical density, as measured by spectrophotometer milligrams per O.D. unit per liter mg/Q.D.* L. expression in terms of mg of protein per O.D. unit per liter.

Purification of the recombinant human ob polypeptide

25 Recombinant human protein may be purified using methods similar to those used for purification of recombinant murine protein, as in Example 13, above. For preparation of recombinant human OB polypeptide, step 8 was performed by adjusting the pH of the supernatant from step 7 to pH 5.0, and loading this on to a CM Sepharose fast flow column. The 20 column volume salt gradient was performed at 20 mM NaOAC, pH 5.5, 0M to 0.5 M NaCl. Step 9 was performed by diluting the CM Sepharose pool four-fold with water, and adjusting the pH to 7.5. This mixture was made to 0.7 M ammonium sulfate. A twenty column volume reverse salt gradient was done at 5 mM NaOAC, pH 5.5, 0.2 M to 0M ammonium sulfate. Otherwise, the above steps were identical.

EXAMPLE 15: Dose Response Studies

An additional study demonstrated that there was a dose response to continuous administration of OB protein. In this study, wild-type mice (non-obese, CD-1 mice,

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weighing 35-40 g) were administered recombinant murine OB protein using methods similar to Examples 12 and 13. The results were as follows (with % body weight lost as compared to baseline, measured as above):

Table 10: Dose Response With Continuous Administration

DOSE	TIME	% REDUCTION IN BODY WEIGHT
0.03 mg/kg/day	Day 2	3.5%
1 mg/kg/day	Day 2	7.5%
l mg/kg/day	Day 4	14%

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As can be seen, increasing the dose from 0.03 mg/kg/day to 1 mg/kg/day increased the weight lost from 3.5% to 7.5%. It is also noteworthy that at day 14, the 1 mg/kg/day dosage resulted in a 14% reduction in body weight.

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EXAMPLE 16: Effects of Leptin on Body Composition of ob/ob Mice

C57Bl/6J ob/ob 16 week old mice were treated with 5 μg/g/day of murine leptin, vehicle, or received no treatment for 33 days. In a second experiment, 7 week old 20 ob/ob mice were treated with 10 μg/g/day of human leptin, murine leptin, or vehicle for 12 days. The mice were sacrificed and total body weight, body composition, insulin levels, and glucose levels were evaluated. The data from these experiments are reported in Table 11.

Table 11

Body Weight, Composition, Insulin Levels, and Glucose Levels of Treated Mice

Treatment Group	16 Wee	16 Week Old Mice, 5 µg/g/day leptin	leptin	7 Weel	Wock Old Mice, 10 µg/g/day leptin	leptin
Trestment	Murine leptin	Vehicle	Control	Human Leptin	Murine Leptin	Vehicle
Total Body Weight	31.90±2.8	64.10±4.5	67.50±6.2	31.00±1.3	33.40±2.4	42.70±1.5
Fat Total(g) %	9.10±1.7 28.40±3.4	38.30±4.0 59.70±2.1	40.87±6.1			
Lean Mass Total (g) %	6.80±1.0 21.30±1.7	7.60±0.4 11.90±1.2	7.73±0.5			
Water Total (g)	16.00±0.8 50.30±4.2	18.20±0.7 28.40±1.0	18.90±1.0 28.10±2.2			
Insulin (UIU/nd)	<2.0	<2.0	<2.0	<2.0	<2.0	21.4
Glucose (mg/d)	170.0±20.9	337±30.3	317.5±51.0	258.3±26.8	320.0±44.0	789.0±152.1

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The body composition data demonstrate the effect of leptin on three compartments of the body: fat mass, lean body mass, and water mass. The date indicate that leptin significantly decreases body fat mass and has a marginal effect on lean body mass. However, the effects on lean body mass were not statistically significant.

Comparison of the insulin and glucose levels in leptin treated and control (untreated) mice indicates that leptin reduces blood sugar and insulin levels, and thus ameliorates

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these indicia of diabetes.

EXAMPLE 17: High Dose Effects of Leptin on Wild-type Mice

Lean controls of the *ob/ob* mice (C57B1/6J+/?) were injected once a day i.p. with 10 μg/g murine leptin or vehicle (PBS), and body weight and food intake were measured over the next two weeks. There was a significant decrease in body weight from day 4 onward and a significant decrease in food intake for the first week. However, after one week, the levels of food intake became indistinguishable between both groups of mice. The animals were sacrificed at the end of the two weeks and body composition was determined. The results of the body composition analysis are shown in Table 12. The data show a decrease in body fat of the animals receiving leptin versus the animals receiving PBS.

Body Composition and Weight of Wildtype (+/?) Mice

9. MASS % Total 10.178 7.044 7.044 11.65% 5.10 28.93% 12.00 11.65% 5.40% 12.00 12.00 14 4.65% 5.16 28.19% 12.00 14 4.65% 5.16 28.19% 12.00 16 2.51% 4.96 28.19% 12.00 16 2.51% 4.96 28.19% 11.30 17 2.28% 4.31 28.83% 11.00 18 1.75% 4.67 28.29% 11.00 18 4.53 2.83% 11.00 18 4.67 28.29% 11.00 19 4.67 28.29% 11.00 10 4.67 28.29% 11.00 10 4.67 28.29% 11.00 10 4.74 4.51 25.59% 11.00 10 4.75 2.59% 11.00 10 4.72 2.5	GROUP	C\$7B1/6J	BODY WT	FAT		LEAN RODY		WATER	
1, 17, 2, 0, 20				Total	₩.	MASS Total	8 ¢	Total	₩.
2 20.5 1.65% 5.41 2.64% 1.20%	Protein	-	17.3	0.30	1.71%	5.00	28.93%	2.0	60 35 0
1		2	20.5	2.39	11.65%	5.41	26.40%	12.8	69.36.8
18.3 0.84 4.63\$ 5.16 28.17\$ 12.30 18.7 0.34 2.51\$ 4.96 28.03\$ 12.30 18.7 2.56 13.72\$ 4.96 28.80\$ 12.30 18.7 2.36 13.72\$ 4.96 28.80\$ 11.30 18.8 16.4 0.23\$ 5.05\$ 4.67 28.23\$ 11.60 19 16.5 0.83 5.05\$ 4.67 28.23\$ 11.60 10 14.9 0.93 5.04\$ 4.87 27.73\$ 11.60 10 14.9 0.93 5.04\$ 4.87 27.73\$ 11.60 11 18.8 1.30 6.93\$ 5.00 26.83\$ 11.20 12 18.6 2.37 12.34\$ 4.61 23.23\$ 11.30 13 18.6 2.35 12.34\$ 4.61 23.23\$ 11.30 14 19.6 3.39 19.34\$ 4.61 23.23\$ 11.30 15 17.3 1.96 11.32\$ 4.45 23.23\$ 11.30 18 20.6 4.16 20.19\$ 4.72 25.64\$ 11.30 19 17.7 1.08 6.13\$ 4.72 25.64\$ 11.30 10 17.7 1.28 12.09\$ 4.75 25.34\$ 11.66 10 11 1 1 1 1 1 1 1 10 10		۴.	6.91	0.34	1.99%	4.76	28.19%	2 2	80.10
1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1,		4	18.3	0.84	4.62%	5.16	28.17%	12.30	67.02.8
18.7 13.7 13.7 13.8 4.84 2.8.6 11.50 8 16.4 0.29 1.139 4.51 2.8.6 11.50 9 16.5 0.29 1.139 4.51 28.39 11.00 10 14.9 0.83 5.05 4.67 28.29 11.00 11 18.8 1.30 6.93 5.04 4.87 27.79 11.00 12 12 12 12.6 2.35 2.35 2.35 11.00 13 18.0 2.29 12.45 4.61 25.39 11.20 14 19.6 2.39 12.45 4.61 25.39 11.20 15 17.0 1.34 4.61 25.39 11.20 16 17.3 1.96 11.32 4.54 25.35 11.20 18 20.6 4.16 20.19 4.94 25.38 11.20 19 17 1.08 6.13 4.72 2.64 11.20 10 10 10 10 2.29 12.50 11.20 10 10 10 2.29 12.45 2.53 2.53 11.20 10 10 10 2.30 2.30 2.30 2.30 11 11 11 4.30 4.75 2.54 4.30 2.30 12 3.30 3.30 3.30 3.30 3.30 3.30 12 3.30 3.30 3.30 3.30 3.30 3.30 12 3.30 3.30 3.30 3.30 3.30 3.30 12 3.30 3.30 3.30 3.30 3.30 3.30 3.30 12 3.30 3.30 3.30 3.30 3.30 3.30 3.30 13 3 3 3 3 3 3 3 3 3		S	17.7	0.44	2.51%	4.96	28.00%	12.30	60 40 G
15.7 0.37 2.38\$ 4.53 28.85\$ 10.80 9 16.5 0.83 1.39\$ 4.67 28.85\$ 10.80 10 14.9 0.83 5.05\$ 4.67 28.29\$ 11.60 11 18.8 1.30 6.93\$ 5.04\$ 4.87 27.79\$ 11.62 11 18.8 1.30 6.93\$ 5.00 26.88\$ 12.50 12 13.6 2.17 12.34\$ 4.61 23.53\$ 11.30 13 18.0 2.39 13.34\$ 4.61 23.53\$ 11.30 14 19.6 3.79 19.34\$ 4.61 23.53\$ 11.30 15 17 1.38 1.35\$ 4.46 23.53\$ 11.30 16 17.3 1.36 1.35\$ 4.45 23.53\$ 11.30 17 19.3 1.38 7.12\$ 5.00 26.45\$ 11.50 18 20.6 4.16 20.19\$ 4.72 26.64\$ 11.50 19 17 1.38 6.13\$ 4.73 25.44\$ 11.50 10 17 1.38 1.20\$ 4.75 25.44\$ 11.66 10 11 1 1 6.64\$ 1.30 11 1 1 6.64\$ 1.30 12 13 13 12.09\$ 4.75 25.54\$ 11.66 13 14.50 14.50 14.50 14.50 14 15 15 15 15 15 15 15 15		çı	18.7	2.56	13.72%	4.84	25.86%	11.30	60.43
10 16.4 0.29 1.79\$, 4.51 27.48\$, 11.60 10 16.5 0.83 5.05\$, 4.67 28.29\$, 11.60 11 18 1.30 5.04\$, 4.87 27.79\$, 11.62 12 13 1.30 6.93\$, 5.06 26.39\$, 11.62 13 18 1.30 6.93\$, 4.61 23.59\$, 11.30 14 19 1.30 1.34\$, 4.61 23.59\$, 11.30 15 18 2.35 1.34\$, 4.61 23.59\$, 11.30 16 17.3 1.36 1.35\$, 4.54 23.59\$, 11.30 17 1.30 1.34\$, 4.54 23.59\$, 11.30 18 20.6 4.16 20.19\$, 4.72 26.64\$, 11.30 19 17 1.38 6.13\$, 4.72 26.64\$, 11.30 10 17 1.38 2.12\$, 4.54 23.99\$, 11.50 10 17 1.38 2.12\$, 4.54 23.99\$, 11.50 11 11 11 11 11 11 11		٠, ١	15.7	0.37	2.38%	4.53	28.83%	10.80	A8 79 %
16.5 0.83 5.05\$ 4.67 28.29\$ 11.00 14.9 0.83 5.05\$ 4.67 28.29\$ 11.00 15.40 0.80 4.52\$ 0.30 1.05\$ 0.74 1		×	16.4	0.29	1.79%	4.51	27.48%	11.60	70.73%
10 4.5 1.1 1.8 1.0 4.5 1.0 4.5 1.0 4.5 1.0 4.5 1.0 4.5 1.0 4.5 1.0 4.5 4.8 1.0 4.5 4.8 1.0 4.5 4.8 1.0 4.5		s :	16.5	0.83	5.05%	4.67	28.29%	11.00	86.67%
Nag 17.3 0.93 5.048 4.87 27.798 11.62 11 18.8 1.30 6.938 5.30 1.058 0.74 12 11.6 1.30 6.938 5.00 25.98 11.50 12 11.6 2.17 12.48 4.53 25.38 11.50 13 18.6 2.17 12.48 4.61 25.59 11.10 14 19.6 3.73 19.34\$ 4.61 25.59\$ 11.20 15 18.6 2.35 11.32\$ 4.74 25.59\$ 11.20 16 17.3 1.96 3.13 4.45 25.59\$ 11.20 16 17.3 1.26\$ 4.75 25.59\$ 11.20 17 19.3 1.13\$ 4.54 25.59\$ 11.20 18 20.6 1.13\$ 4.72 26.64\$ 11.50 20 19.5 1.26\$ 2.13\$ 12.28 12.20 12.30		2	14.9					10.40	69.80%
Sid Dev. 16 0.90 4.524 0.30 1.057 0.74 1 8.8 1.30 6.934 5.00 26.584 12.50 1 18.8 1.30 6.934 5.00 26.584 12.50 1 18.0 2.17 12.45 4.61 25.534 11.50 1 19.6 2.35 12.45 4.61 23.534 11.30 1 1 1 1 1.26 1.35 4.45 25.534 11.50 1 1 1 1 1.26 1.35 4.45 25.534 11.50 1 1 1 1 1 1 1 1 1 1		Avg.	17.3	0.93	5.04%	4.87	77 79 €	517	75.5
11 18.8 1.30 6.93% 5.00 26.58% 12.50 12.51 13.45 13.45 4.51 21.77 13.45 13.45 14.61 23.59% 11.10 11.10 13.45 4.61 23.59% 11.10 11.10 13.45 4.61 23.59% 11.10 11.10 13.45 4.61 23.59% 11.10 13.45 4.61 23.59% 11.10 13.45 4.45 23.59% 11.20 13.50 1		Std. Dev.	9:1	06:0	4.52%	0.30	1.05%	77.0	25.5%
1 18.8 1.30 6.93 8 5.00 2.58 1.250 1	Vehicle	:						0.7	3.32.8
17.6 2.17 11.34% 4.53 23.73% 10.90 18.6 2.37 12.44% 4.61 23.53% 11.10 18.6 2.35 12.65% 4.61 23.53% 11.30 17.3 1.36 1.12% 4.54 25.53% 11.50 17.5 4.61 22.53% 12.60% 12.50 17.5 4.61 2.019% 4.54 25.53% 11.50 17.5 4.61 20.19% 4.72 25.64% 11.50 18.7 2.28 12.99% 4.75 25.54% 11.66 18.7 2.28 12.99% 4.75 25.54% 11.66 18.7 2.28 2.29% 4.75 25.54% 11.66 18.7 2.28 2.29% 4.75 25.54% 11.66 18.7 2.28 2.29% 4.75 25.54% 11.66 18.7 2.28 2.29% 4.75 2.54% 11.66 18.8 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 4.75 2.54% 11.66 18.9 2.29% 2.29% 2.29% 11.66 18.9 2.29% 2.29% 2.29% 2.29% 11.66 18.9 2.29% 2.29% 2.29% 2.29% 2.29% 2.29% 18.9 2.29% 2.	· cincie	= :	8.	06.	6.93%	2:00	26.58%	12.50	66.49%
18 0 2.79 12.4% 461 23.59% 11.10 11.0 11.0 11.0 12.4% 461 23.59% 11.10 12.0% 4.75 23.59% 11.20 12.0% 4.75 23.59% 11.20 11.		7 :	17.6	2.17	12.34%	4.53	25.73%	10.90	61 93%
19.6 3.79 19.4% 4.61 23.25% 11.20 11.20 12.65% 4.75 23.25% 11.20 11.20 12.65% 4.75 23.25% 11.20 11.20 12.2		2 :	0.8	2.29	12.74%	4.61	25.59%	11.10	61.67%
12.6 2.35 1.26 4.75 25.25 11.50 11.50 11.50 11.20		4 7	9.6	3.79	19.34%	4.61	23.52%	11.20	57.14%
17.3 1.96 11.32% 4.54 26.55% 10.80 20.6 4.16 20.19% 4.94 23.99% 11.50 17.7 1.08 6.13% 4.72 26.64% 11.50 18.7 2.38 12.09% 4.75 25.54% 11.66 18.1 2.38 12.09% 6.45 25.54% 11.66 18.1 1.07 6.66% 6.45 11.66 18.2 2.38 2.39% 11.66 18.3 2.38 2.39% 11.66 20.5 2.54% 2.54% 2.54% 11.66 20.5 2.54% 2.54% 2.54% 2.54% 2.54% 20.5 2.54% 2.54% 2.54% 2.54% 2.54% 20.5 2.54% 2.54% 2.54% 2.54% 2.54% 20.5 2.54% 2.54% 2.54% 2.54% 2.54% 2.54% 20.5 2.54% 2.54% 2.54% 2.54% 2.54% 2.54% 20.5 2.54% 2.54		2 3	9.9	2.35	12.65%	4.75	25.52%	11.50	61.83%
1.38 7.12% 5.02 25.04% 12.90 2.0		2 5		96	11.32%	4.54	26.25%	10.80	62.43%
7.00 4.16 20.19% 4.94 23.98% 11.50 1			. v. v.	86.	7.12%	5.02	26.04%	12.90	66.84%
17 1.08 6.13% 4.72 2.6.64% 11.90 11.90 12.30 12.30 12.09% 4.75 2.5.44% 11.66		9 9	9.07	4.16	20.19%	4.94	23.98%	11.50	55.83%
18.7 2.28 12.09% 4.75 25.54% 11.66 11.66		<u> </u>		80.	6.13%	4.72	26.64%	96:11	67.23%
18.7 2.28 12.09% 4.75 25.54% 11.66		83	6.61					12.30	63.08%
00.11		Avg.	18.7	2.28	12.09%	4.75	25 54 95	77 11	20,00
		Std. Dev.	_	101	2000	9		8.1	8 C+ 70

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A second experiment showed the effects of twice a day i.p. injections of 12.5 µg/g of murine leptin on wild-type C57B1/6J mice. There was a significant decrease in body weight and food intake associate with twice daily injections of the polypeptide. For this experiment, the animals were placed in metabolic chambers. Food consisted of a powdered Purina #5001 chow diet. This diet differed from earlier experiments, which used the diet consisting of chow diet, tapioca, and water. Thus the food used in the metabolic chambers had a higher caloric content, which explains why the amount of food consumed differs from those animals on the water-containing diet.

10 The following is a list of references related to the above disclosure and particularly to the experimental procedures and discussions.

Bahary et al., Genomics, 11:33-47 (1991).

15 Bahary et al., Genomics, 13:761-769 (1992).

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Bahary et al., Molecular mapping of mouse chromosomes 4 and 6: Use of a flow-sorted Robertsonian chromosome (1991).

Blank et al., Mammalian Genome, 1:s51-s78 (1991).

Bogardus et al., Annals of the New York Academy of Sciences, 630:100-115 (1991).

25 Friedman et al., Mammalian Genome, 1:130-144 (1991).

Harris, FASEB J., 4:3310-3318 (1990).

Jacobowitz et al., N. Engl. J. Med., 315:96-100 (1986).

Kessey, in *Obesity*, pp. 144-166, Stunkard ed., Philadelphia, W.B. Sauders Co. (1980).

165

Kessey et al., Ann. Rev. Psychol., 37:109-133.22 (1986).

Leibel et al., "Genetic variation and nutrition in obesity: Approaches to the molecular genetics of obesity", in *Genetic Variation and Nutrition*, pp. 90-101.1, Simopoulos and Childs eds., S. Karger, Basel (1990).

Siegel et al., Cytogenet. Cell Genet., 61(3):184-185 (1992).

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein

15 Various references are cited throughout this specification, each of which is incorporated herein by reference in its entirety.

The invention as claimed is enabled in accordance with the above specification and readily available references and starting materials. Nevertheless, Applicants have on 20 August 9, 1995, made the following deposits with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852-1178, U.S.A. in accord with the regulations of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure: E. coli H14 harboring plasmid pETH14, accession No. 69880; and E. coli M9 harboring plasmid pETH9, accession No. 69879.

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SECUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: THE ROCKEFELLER UNIVERSITY
- (ii) TITLE OF INVENTION: MODULATORS OF BODY WEIGHT, CORRESPONDING NUCLEIC ACIDS AND PROTEINS, AND DIAGNOSTIC AND THERAPEUTIC USES THEREOF
- (iii) NUMBER OF SEQUENCES: 99
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSER: Klauber & Jackson
 - (B) STREET: 411 Hackensack Avenue
 - (C) CITY: Hackensack
 - (D) STATE: New Jersey
 - (E) COUNTRY: USA
 - (F) ZIP: 07601
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/483,211
 - (B) FILING DATE: June 7, 1995
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/438.431
 - (B) FILING DATE: May 10, 1995
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/347,563
 - (B) FILING DATE: November 30, 1994
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/292,345
 - (B) FILING DATE: August 17, 1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Jackson Esq., David A.
 - (B) REGISTRATION NUMBER: 26,742

(C) REFERENCE/DOCKET NUMBER: 600-1-087 PCT

(1x) TELECOMMUNICATION INFORMATION:

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	(xi)	SEÇ	UENC	E DE	SCRI	PTIC	N: S	EQ I	D NO	1:1:						
GGA	rrece	rige	TCCA	GCAG	CT G	CAAG	GTGC	A AG	AAGA	AGAA	GAT	CCCA	GGG	AGGA	AA	56
ATG	TGC	TGG	AGA	ccc	CTG	тст	caa	Tell	CTIC	тсс	C-1-1	- TYC	TCC		CTG	
Met	Сув	Trp	Arg	Pro	Leu	Cvs	Ara	Phe	Leu	Tro	Len	Trn	Ser	Three	Leu	104
1		-	-	5			5		10				501	15	Deu	
TCT	TAT	GTT	CAA	GCA	GTG	CCT	ATC	CAG	AAA	GTC	CAG	GAT	GAC	ACC	AAA	152
Ser	Tyr	Val	Gln	Ala	Val	Pro	Ile		Lys	Val	Gln	Asp	Asp	Thr	Lys	
			20					25					30			
ACC	CTC	ATC	AAG	NCC.	P. Jan.	GTC	200	B.C.C	200							
Thr	Leu	Tle	Lys	Thr	Tle	1721	The	AGG	TIO	AAI	BAC	ATT	TCA	CAC	ACG	200
		35	-, -			•	40	AL 9	116	ABII	Авр	45	ser	HIB	Int	
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CAG	TCG	GTA	TCC	GCC	AAG	CAG	AGG	GTC	ACT	GGC	TTG	GAC	TTC	ATT	CCT	248
Glii	Ser	val	ser	Ala	Lys	Gln	Arg	Val	Thr	Gly	Leu	Авр	Phe	Ile	Pro	
	50					55					60					
ccc	بتعلب	CAC			ome											
Glv	Lev	UAC	CCC	ATT	CTG	AGT	TTG	TCC	AAG	ATG	GAC	CAG	ACT	CTG	GCA	296
65	red	HIR	Pro	11 6	70	ser	Leu	ser	Lys		Asp	Gln	Thr	Leu		
0.5					/0					75					80	

GTC TAT CAA CAG GTC CTC ACC AGC CTG CCT TCC CAA AAT GTG CTG CAG Val Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln 85 90 95	34
ATA GCC AAT GAC CTG GAG AAT CTC CGA GAC CTC CTC CAT CTG CTG GCC Ile Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala 100 105 110	39:
TTC TCC AAG AGC TGC TCC CTG CCT CAG ACC AGT GGC CTG CAG AAG CCA Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro 115 120 125	440
GAG AGC CTG GAT GGC GTC CTG GAA GCC TCA CTC TAC TCC ACA GAG GTG Glu Ser Leu Amp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val 130 135 140	488
GTG GCT TTG AGC AGG CTG CAG GGC TCT CTG CAG GAC ATT CTT CAA CAG Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln 145 150 155 160	536
TTG GAT GTT AGC CCT GAA TGC TGA AGTTTCAAAG GCCACCAGGC TCCCAAGA Leu Asp Val Ser Pro Glu Cys * 165	588
ATCATGTAGA GGGAAGAAAC CTTGGCTTCC AGGGGTCTTC AGGAGAAGAG AGCCATGTGC	648
ACACATCCAT CATTCATTTC TCTCCCTCCT GTAGACCACC CATCCAAAGG CATGACTCCA	708
CAATGCTTGA CTCAAGTTAT CCACACAACT TCATGAGCAC AAGGAGGGGC CAGCCTGCAG	768
AGGGGACTCT CACCTAGTTC TTCAGCAAGT AGAGATAAGA GCCATCCCAT CCCCTCCATG	828
TCCCACCTGC TCCGGGTACA TGTTCCTCCG TGGGTACACG CTTCGCTGCG GCCCAGGAGA	888
GGTGAGGTAG GGATGGGTAG AGCCTTTGGG CTGTCTCAGA GTCTTTGGGA GCACCGTGAA	948
GGCTGCATCC ACACAGGT GGAAACTCCC AAGCAGCACA CGATGGAAGC ACTTATTTAT	1008
TTATTCTGCA TTCTATTTTG GATGGATCTG AAGCAAGGCA TCAGCTTTTT CAGGCTTTGG	1068
GGGTCAGCCA GGATGAGGAA GGCTCCTGGG GTGCTGCTTT CAATCCTATT GATGGGTCTG	1128
CCCGAGGCAA ACCTAATTIT TGAGTGACTG GAAGGAAGGT TGGGATCTTC CAAACAAGAG	1188
TCTATGCAGG TAGCGCTCAA GATTGACCTC TGGTGACTGG TTTTGTTTCT ATTGTGACTG	1248
ACTOTATOCA AACACGTTTG CAGCGGCATT GCCGGGAGCA TAGGCTAGGT TATTATCAAA	1308
AGCACATCAA TITTGTCAAG TGTAATATGT ATCTATGTGC ACCTGAGGGT AGAGGATGTG	1368
TTAGAGGGAG GGTGAAGGAT CCGGAAGTGT TCTCTGAATT ACATATGTGT GGTAGGCTTT	1428
TCTGAAAGGG TGAGGCATTT TCTTACCTCT GTGGCCACAT AGTGTGGCTT TGTGAAAAGG	1488
ACAAAGGAGT TGACTCTTTC CGGAACATTT GGAGTGTACC AGGCACCCTT GGAGGGGCTA	1549

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AAGC	TACAGG	CCTTTTGTTC	GCATATTGCT	GAGCTCAGG	G AGTGAGGGC	CCACATTTGA	1608
GACA	GTGAGC	CCCAAGAAA	GGGTCCCTGC	G TGTAGATCT	CAAGGTTGT	CAGGGTTGAT	1668
CTCA	CAATGC	GTTTCTTAAC	CAGGTAGACO	TTTGCATGC	AATATGTGGT	TCTCATCTGA	1728
TTGG	TCATC	CAAAGTAGAA	CCCTGTCTCC	CACCCATTC	GTGGGGAGTT	TTGTTCCAGT	1788
GGGA	ATGAGA	AATCACTTAG	CAGATGGTCC	TGAGCCCTGG	GCCAGCACTG	CTGAGGAAGT	1848
GCCA	GGCCC	CAGGCCAGGC	TGCCAGAATI	GCCCTTCGGC	CTGGAGGATG	AACAAAGGGG	1908
CTTG	GTTTT	TCCATCACCC	CTGCACCCTA	TGTCACCATC	AAACTGGGGG	GCAGATCAGT	1968
GAGA	GACAC	TTGATGGAAA	GCAATACACT	TTAAGACTGA	GCACAGTTTC	GTGCTCAGCT	2028
CTGT	TGGTG	CTGTGAGCTA	GAGAAGCTCA	CCACATACAT	ATAAAAATCA	GAGGCTCATG	2088
TCCCT	GTGGT	TAGACCCTAC	TCGCGGCGGT	GTACTCCACC	ACAGCAGCAC	CGCACCGCTG	2148
GAAGT	ACAGT	GCTGTCTTCA	ACAGGTGTGA	AAGAACCTGA	GCTGAGGGTG	ACAGTGCCCA	2208
GGGGA	ACCCT	GCTTGCAGTC	TATTGCATTT	ACATACCGCA	TTTCAGGGCA	CATTAGCATC	2268
CACTO	CTATG	GTAGCACACT	GTTGACAATA	GGACAAGGGA	TAGGGGTTGA	CTATCCCTTA	2328
TCCAA	AATGC	TTGGGACTAG	AAGAGTTTTG	GATTTTAGAG	TCTTTTCAGG	CATAGGTATA	2388
TTTGA	GTATA	TATAAAATGA	GATATCTTGG	GGATGGGGCC	CAAGTATAAA	CATGAAGTTC	2448
ATTTA	TATTT	CATAATACCG	TATAGACACT	GCTTGAAGTG	TAGTTTTATA	CAGTGTTTTA	2508
AATAA	CGTTG	TATGCATGAA	AGACGTTTTT	ACAGCATGAA	CCTGTCTACT	CATGCCAGCA	2568
TCAA	AAACC	TTGGGGTTTT	GGAGCAGTTT	GGATCTTGGG	TTTTCTGTTA	AGAGATGGTT	2628
GCTT.	ATACC	TAAAACCATA	ATGGCAAACA	GGCTGCAGGA	CCAGACTGGA	TCCTCAGCCC	2688
GAAG	TGTGC	CCTTCCAGCC	AGGTCATACC	CTGTGGAGGT	GAGCGGGATC	AGGTTTTGTG	2748
TGCT	AAGAG.	AGGAGTTGGA	GGTAGATTTT	GGAGGATCTG	AGGGC		2793

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 168 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
 (A) DESCRIPTION: Murine ob polypeptide

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(- i)	CECKTERCE	DESCRIPTION.	CEO	TD	NO - 2 :

Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu 1 5 10 15

Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys 20 25 30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr 35 40 45

Gln Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro

Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala 65 70 75 80

Val Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln 85 90 95

Ile Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala 100 105 110

Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro 115 120 125

Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val 130 135 140

Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln 145 150 150

Leu Asp Val Ser Pro Glu Cys * 165

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 700 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

- (A) DESCRIPTION: Human ob cDNA where N represents any nucleotide
- (111) HYPOTHETICAL: NO
- (1V) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human

171

(A) NAME/KEY: CDS

(B) LOCATION: 46..546

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NNN	GNN	FTTG	CAAC	GCCC	CAA C	AAGO	CCA	N NT	rccro	GGA	A GGZ		ATG (Met i			54
		Leu					Trp					Lev			GTC Val	102
	Ala					Lys					Thr				ATC Ile 35	150
AAG Lys	ACA Thr	Ile	GTC Val	Thr 40	Arg	ATC Ile	AAT Asn	GAC Asp	ATT Ile 45	TCA Ser	CAC His	ACG	Gln	TCA Ser 50		198
														Leu	CAC His	246
CCC Pro	ATC Ile	CTG Leu 70	ACC Thr	TTA Leu	TCC Ser	AAG Lys	ATG Met 75	GAC Asp	CAG Gln	ACA Thr	CTG Leu	GCA Ala 80	GTC Val	TAC Tyr	CAA Gln	294
CAG Gln	ATC Ile 85	CTC Leu	ACC Thr	AGT Ser	ATG Met	CCT Pro 90	TCC Ser	AGA Arg	AAC Asn	GTG Val	ATC Ile 95	CAA Gln	ATA Ile	TCC Ser	AAC Asn	342
				CTC Leu												390
AGC Ser	TGC Cys	CAC His	TTG Leu	CCC Pro 120	TGG Trp	GCC Ala	AGT Ser	GGC Gly	CTG Leu 125	GAG Glu	ACC Thr	TTG Leu	GAC Asp	AGC Ser 130	CTG Leu	438
GGG Gly	GGT Gly	Val	CTG Leu 135	GAA Glu	GCT Ala	TCA Ser	GGC Gly	TAC Tyr 140	TCC Ser	ACA Thr	GAG Glu	GTG Val	GTG Val 145	GCC Ala	CTG Leu	486
AGC :	Arg	CTG Leu 150	CAG Gln	GGG :	TCT Ser	Leu ·	CAG Gln 155	GAC Asp	ATG Met	CTG Leu	Trp	CAG Gln 160	CTG Leu	GAC Asp	CTC Leu	534
Ser 1	CCT Pro	GGG Gly	TGC Cys	TGAG	GCCT	T GA.	AGGT	CACT	CII	CCTG	CAA	GGAC	TNAC	GT		585

PCT/US95/10479 WO 96/05309

										172							
AAT	GGGA	AGG	AACT	CTGG	TT T	CCAG	GTAT	с тс	CAGG	ATTG	AAG	agca	TTG	CATG	GACAC	с е	645
CCT	TATC	CAG	GACT	CTGT	CA A	TTTC	ccrg	A CT	ccrc	TAAG	CCA	CTCT	TCC	aaag	G	7	700
(2)	INF	ORMA	TION	FOR	SEQ	ID	NO : 4	;									
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 167 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear																	
(ii) MOLECULE TYPE: protein (A) DESCRIPTION: Human ob polypeptide																	
	(vi)	ORIG	INAL	sou	RCE:	Hum	an									
	(:	xi)	SEQU	ENCE	DES	CRIP	TION	: SE	Q ID	NO:	4 :						
Met 1	His	Trp	Gly	Thr 5	Leu	Сув	Gly	Phe	Leu 10		Leu	Trp	Pro	Tyr 15			
Phe	Tyr	Val	Gln 20	Ala	Val	Pro	Ile	Gln 25	Lys	Val	Gln	Авр	As p 30		Lys		
Thr	Leu	Ile 35	Lys	Thr	Ile	Val	Thr 40	Arg	Ile	Asn	Asp	Ile 45	Ser	His	Thr		
Gln	Ser 50	Val	Ser	Ser	Lys	Gln 55	Lys	Val	Thr	Gly	Leu 60	Asp	Phe	Ile	Pro		
Gly 65	Leu	His	Pro	Ile	Leu 70	Thr	Leu	Ser	Lys	Met 75	Asp	Gln	Thr	Leu	Ala 80		
Val	Tyr	Gln	Gln	11e 85	Leu	Thr	Ser	Met	Pro 90	Ser	Arg	Asn	Val	Ile 95	Gln		
Ile	Ser	Asn	Asp 100	Leu	Glu	Asn	Leu	Arg 105	Asp	Leu	Leu	His	Val 110	Leu	Ala		
Phe	Ser	Lув 115	Ser	Сув	His	Leu	Pro 120	Trp	Ala	Ser	Gly	Leu 125	Glu	Thr	Leu		
Авр	Ser 130	Leu	Gly	Gly	Val	Leu 135	Glu	Ala	Ser	Gly	Tyr 140	Ser	Thr	Glu	Val		
Val 145	Ala	Leu	Ser	Arg	Leu 150	Gln	Gly	Ser	Leu	Gln 155	Двр	Met	Leu	Trp	Gln 160		

Leu Asp Leu Ser Pro Gly Cys 165

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(2) INFORMATION FOR SEC ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 166 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (A) DESCRIPTION: Murine ob polypeptide lacking Gln at position 49
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: Murine
- (xi) SEQUENCE DESCRIPTION: SEO ID NO:5:

Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu 1 5 10 15

Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys \$20\$ \$25\$ \$30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr 35 40 45

Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly 50

Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Val 65 70 75 80

Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln Ile 85 90 95

Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala Phe 100 105 110

Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu 115 120 125

Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val Val 130 140

Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu 145 \$150\$

Asp Val Ser Pro Glu Cys

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 167 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) Description: Human ob polypeptide lacking Gln at position 49

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEO ID NO:6:

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu

1 5 10 15

Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
20 25 30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr 35 40 45

Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly 50 60

Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val 65 70 75 80

Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln Ile 85 90 95

Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe 100 105 110

Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp 115 120 125

Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val

Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu 145 150 150 160

Asp Leu Ser Pro Gly Cys 165

(2) INFORMATION FOR SEQ ID NO:7: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 175 base pairs (B) Type: nucleic acid (C) STRANDENNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (A) DESCRIPTION: exon 2G7 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG 1: ACACCAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDENNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7 ((iii) HYPOTHETICAL: NO
(A) LENGTH: 175 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (A) DESCRIPTION: exon 2G7 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG ACACCAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(A) DESCRIPTION: exon 2G7 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG ACACCAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(iv) ANTI-SENSE: NO (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG ACACCAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (ii) LENGTH: 18 base pairs (ii) LENGTH: 18 base pairs (ii) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (iii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG ACACCAAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEOMESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
GTGCAAGAAG AAGAAGATCC CAGGGCAGGA AAATGTGCTG GAGACCCCTG TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC TATCCAGAAA GTCCAGGATG ACACCAAAAAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (ii) LENOTH: 18 base pairs (ii) TYPE: nucleic acid (C) STRANDEDNESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (ii) PCR 5' primer for exon 2G7
NOTEGNITIE GECCEATECTE TETTATETIC AAGCAGTECE TATECAGAAA GECCAGGATE ACACCAAAAG CETCATCAAG ACCATTGEA NEAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYFE: nucleic acid (C) STRANDEDNESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
ACACCAARAG CCTCATCAAG ACCATTGTCA NCAGGATCAC TGANATTTCA CACACG (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) FCR 5' primer for exon 2G7
(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDENNESS: Single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) PCR 5' primer for exon 2G7
(A) PCR 5' primer for exon 2G7
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
CCAGGGCAGG AAAATGTG 18
(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE: nucleat acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (primer) (A) PCR 3' primer for exon 2G7

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEOUENCE DESCRIPTION: SEQ ID NO:9:

CATCCTGGAC TTTCTGGATA GG

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- (2) INFORMATION FOR SEO ID NO:10:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 amino acids
 - (B) TYPE: amino acid (D) TOPOLOGY: linear

 - (ii) MOLECULE TYPE: peptide (A) DESCRIPTION: putative N-terminal signal peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu 10

Ser Tyr Val Gln Ala Val Pro 20

- (2) INFORMATION FOR SEQ ID NO:11:
 - (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 287 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: circular
 - (ii) MOLECULE TYPE: DNA (plasmid) (A) DESCRIPTION: pET-15b expression vector
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (ix) FEATURE:
 - (A) NAME/KEY: T7 promoter
 - (B) LOCATION: 20..37
 - (1x) FEATURE:
 - (A) NAME/KEY: lac op rator
 - (B) LOCATION: 39..64

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(ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 108243	
(ix) FEATURE: (A) NAME/KEY: His-Tag (B) LOCATION: 123137	
(ix) FEATURE: (A) NAME/KEY: Thrombin cleavage site (B) LOCATION: 184196	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:	
AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
TTCCCCTCTA CAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC AGC Met Gly Ser 1	116
AGC CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC Ser His His His His His Ser Ser Gly Leu Val Pro Arg Gly Ser 5 10 15	164
CAT ATG CTC GAG GAT CCC GCT GCT AAC AAA GCC CGA AAG GAA GCT GAG His Met Leu Glu Asp Pro Ala Ala Asn Lys Ala Arg Lys Glu Ala Glu 20 25 30 35	212
TTG GCT GCT GCC ACC GCT GAG CAA TAA CTA G CATAACCCCT TGGGGCCTCT Leu Ala Ala Thr Ala Glu Gln $$	263
AAACGGGTCT TGAGGGGTTT TTTG	287
(2) INFORMATION FOR SEQ ID NO:12:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 45 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: protein	
(x1) SEQUENCE DESCRIPTION: SEQ ID NO:12:	
Met Gly Ser Ser His His His His His Ser Ser Gly Leu Val Pro	
Arg Gly Ser Hi s Met Le u Glu A s p Pro Al a Ala As n Lys Ala Ar g Lys 20 25 30	
Glu Ala Glu Leu Ala Ala Ala Thr Ala Glu Gln 35 40	

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(2) INFORMATION FOR SEQ ID NO:13:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Murine 5' primer	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
CTTATGTTCA TATGGTGCCG ATCCAGAAAG TC	32
(2) INFORMATION FOR SEQ ID NO:14: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Murine 3' primer	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: Yes	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14: TCCCTCTACA TATGTCTTGG GAGCCTGGTG GC	32
(2) INFORMATION FOR SEQ ID NO:15:	
(1) SEQUENCE CHARACTERISTICS: (A) LENGTH, 31 base pairs (B) TTPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: DNA (primer)
(A) DESCRIPTION: Human 5' primer

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(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
TCTATGTCCA TATGGTGCCG ATCCAAAAAG TC	32
(2) INFORMATION FOR SEQ ID NO:16:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Human 3' primer	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: Yes	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
TTCCTTCCCA TATGGTACTC CTTGCAGGAA GA	32
(2) INFORMATION FOR SEQ ID NO:17:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 11 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA (A) DESCRIPTION: Splice acceptor site in ob	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(1x) FRATTIDE: (A) NAME/KEY: Splice acceptor site	
(x1) SEQUENCE DESCRIPTION: SEQ ID NO:17:	

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AGCAGTCGGT A

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(2) INFORMATION FOR SEO ID NO:18:

(i) SECUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: peptide
 - (A) DESCRIPTION: ob peptide fragment
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Murine
- (xi) SECUENCE DESCRIPTION: SEO ID NO:18:

- (2) INFORMATION FOR SEQ ID NO:19:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: unknown
 - (ii) MOLECULE TYPE: peptide
 - (A) DESCRIPTION: ob peptide fragment
 - (v) FRAGMENT TYPE: internal
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Murine
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala 1 5 10 15

- (2) INFORMATION FOR SEQ ID NO:20:
 - (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: unknown
 - (ii) MOLECULE TYPE: peptide
 - (A) DESCRIPTION: ob peptide fragment
 - (v) FRAGMENT TYPE: internal

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(vi) ORIGINAL SOURCE:
           (A) ORGANISM: Murine
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
      Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro Glu
                                         10
      Ser Leu Asp
 (2) INFORMATION FOR SEQ ID NO:21:
      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 20 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: unknown
     (ii) MOLECULE TYPE: peptide
          (A) DESCRIPTION: ob peptide fragment
     (v) FRAGMENT TYPE: Carboxyl terminal
    (vi) ORIGINAL SOURCE:
          (A) ORGANISM: Murine
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
     Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln Leu Asp Val
     1
                    5
                                         10
     Ser Pro Glu Cvs
(2) INFORMATION FOR SEQ ID NO:22:
     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 414 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: DNA (genomic)
         (A) DESCRIPTION: portion of the human ob gene including noncoding
           sequence upstream of first exon, coding sequence of first
       exon, and 5' region of first intron
  (ili) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: NO
   (vi) ORIGINAL SOURCE:
```

(A) ORGANISM: Human

(ix) FEATURE:

(A) NAME/KEY: CDS (B) LOCATION: 38..181

(1) SEQUENCE CHARACTERISTICS (A) LENGTH: 48 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear

<pre>(ix) FEATURE: (A) NAME/KEY: 5' region of first intron</pre>	
(B) LOCATION: 182414	
(ix) FEATURE:(A) NAME/KEY: 5' noncoding sequence of the human ob gene f	rom which
the HOB 1gF DNA primer was generated (B) LOCATION: 11. 28	TOM WITTEN
(ix) FEATURE:(A) NAME/KEY: intronic sequence of the human ob gene from	which the
HOB 1gR primer was generated (B) LOCATION: 241260	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:	
GGTTGCAAGG CCCAAGAAGC CCATCCTGGG AAGGAAA ATG CAT TGG GGA ACC CTG	55
Met His Trp Gly Thr Leu 1 5	
TGC GGA TTC TTG TGG CTT TGG CCC TAT CTT TTC TAT GTC CAA GCT GTG	103
Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu Phe Tyr Val Gln Ala Val	
CCC ATC CAA AAA GTC CAA GAT GAC ACC AAA ACC CTC ATC AAG ACA ATT	151
Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys Thr Ile 25 30 35	
GTC ACC AGG ATC AAT GAC ATT TCA CAC ACG GTAAGGAGAG TATGCGGGGA Val Thr Arg Ile Asn Asp Ile Ser His Thr	201
40 45	
CAAAGTAGAA CTGCAGCCAG CCCAGCACTG GCTCCTAGTG GCACTGGACC CAGATAGTCC	261
AAGAAACATT TATTGAACGC CTCCTGAATG CCAGGCACCT ACTGGAAGCT GAGAAGGATT	321
TTGGATAGCA CAGGGCTCCA CTCTTTCTGG TTGTTTCTTN TGGCCCCCTC TGCCTGCTGA	381
GATNCCAGGG GTTAGNGGTT CTTAATTCCT AAA	414
(2) INFORMATION FOR SEQ ID NO:23:	

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(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: N-terminal portion of the human ob protein encoded by first exon

(xi) SEQUENCE DESCRIPTION: SEO ID NO:23:

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu 1 5 10 15

Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
20 25 30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr 35 40 45

- (2) INFORMATION FOR SEC ID NO:24:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 801 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (A) DESCRIPTION: portion of the human ob gene including 3' region of first intron, coding sequence of second exon, and 3' noncoding sequence
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 291..648
 - (ix) FEATURE:
 - (A) NAME/KEY: 3' of first intron
 - (B) LOCATION: 1..290
 - (ix) FEATURE:
 - (A) NAME/KEY: intronic sequence of the human ob gene HOB from which the HOB 2qF primer was generated
 - (B) LOCATION: 250..269
 - (ix) FEATURE:
 - (A) NAME/KEY: 3' noncoding sequence of the human ob gene from which the HOB 2gR DNA primer was generated
 - (B) LOCATION: 707..728

	(xi) SE	OUE	ICE D	ESCR	IPTI	ON:	SEQ	ID N	10:24	:					
CTG	GTTC	TTT	CAGG	AAGA	.ec c	CATG	TAAG	A GA	AAGG	LAATT	GAC	CTAG	GGA	AAAT	TGGCC	r 6
GGG	aagt	GGA	GGGA	ACGG	AT G	GTGT	GGGA	AA A	GCAG	GAAT	CIC	GGAG	ACC	AGCI	TAGAG	3 120
CTT	GGCA	GTC	ACCT	GGGT	GC A	GGAN	ACAA	G GG	CCTG	AGCC	AAA	GTGG	TGA	GGG	GGGTG	3 180
AAG	GAGA	.CAG	CCCA	GAGA	AT G	ACCC	TCCA	T GC	CCAC	GGGG	AAG	GCAG	AGG	GCTC	TGAGA	3 240
CGA	TTCC	TCC	CACA	TGCT	ga g	CACT	TGTT	C TC	CCTC	TTCC	TCC	TNCA	TAG	CAG Gln 1		290
			Lys					Gly					Pro		CTC	344
												Leu			TAC	392
															TCC Ser 50	440
										His					TCT Ser	488
										CTG Leu						536
										TCC Ser						584
										ATG Met						632
			GGG Gly		T G≇	GCC	TTG	AGC	STCAC	CTCT	TCCT	rgcaj	AGG 2	ACTA	GTTAA	688
GGG2	VAGGA	AC 1	CTGC	CITI	c ca	GGTA	тсто	CAC	GATI	rgaa	GAG	ATTO	CA T	rgga	ACCCC	748
TTAT	רבת	GA C	بالملماط	TCA	T TT	CCC	تتالاته	تت.	CTAI	vacc.	ACTO	TTCC	י אני	vec		801

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(2) INFORMATION FOR SEO ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 119 amino acids
 - (B) TYPE: amino acid
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
 - (A) DESCRIPTION: C-terminal portion of the human ob protein encoded by second exon
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
- Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro 1 5 10 15
- Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala $20 \hspace{1cm} 25 \hspace{1cm} 30$
- Val Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln 35 40 45
- Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala 50 55 60
- Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu 65 70 75 80
- Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val 85 90 95
- Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln
 100 105 110

Leu Asp Leu Ser Pro Gly Cys

- (2) INFORMATION FOR SEQ ID NO:26:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: unknown
 - (i1) MOLECULE TYPE: peptide
 - (v) FRAGMENT TYPE: int rnal
 - (vi) ORIGINAL SOURCE:
 (A) ORGANISM: pichia yeast

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Leu Glu Lys Arg Glu Ala Glu Ala

- (2) INFORMATION FOR SEO ID NO:27:
 - (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 4 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: unknown
 - (ii) MOLECULE TYPE: peptide
 - (v) FRAGMENT TYPE: internal
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: pichia yeast
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
 - Glu Ala Glu Ala 1

- (2) INFORMATION FOR SEO ID NO:28:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4 amino acids
 - (B) TYPE: amino acid (D) TOPOLOGY: unknown
 - (ii) MOLECULE TYPE: peptide
 - (v) FRAGMENT TYPE: Internal
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: pichia yeast
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
 - Leu Glu Lys Arg
 - 1
- (2) INFORMATION FOR SEO ID NO:29:
 - (+) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

sequence of the human ob gene

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(A) DESCRIPTION: HOB 1gF DNA primer generated from the 5' noncoding

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(iii) HYPOTHETICAL: NO
      (iv) ANTI-SENSE NO
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
  CCCAAGAAGC CCATCCTG
                                                                           18
  (2) INFORMATION FOR SEQ ID NO:30:
       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: DNA (primer)
            (A) DESCRIPTION: HOB 1gR DNA primer generated from the first
                intronic sequence of the human ob gene
        (iii) HYPOTHETICAL: NO
         (iv) ANTI-SENSE: YES
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:
 GACTATCTGG GTCCAGTGCC
                                                                          20
 (2) INFORMATION FOR SEQ ID NO:31:
      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 20 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: DNA (primer)
           (A) DESCRIPTION: HOB 2gF DNA primer generated from the first
        intronic sequence of the human ob gene
   (iii) HYPOTHETICAL: NO
    (1V) ANTI-SENSE NO
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:
CCACATGCTG AGCACTTGTT
                                                                         20
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(2) INFORMATION	FOR SEQ ID NO:32:			
(A) LE (B) TY (C) ST	E CHARACTERISTICS: NGTH: 22 base pairs PE: nucleic acid RANDEDNESS: single POLOGY: linear			
(ii) MOLECUL (A) DE sequence of the) DNA primer generated	from the 3'	noncoding
(ili) HYPOTHE				
(iv) ANTI-SE				
(xi) SEQUENC	E DESCRIPTION: SEQ	ID NO:32:		
CTTCAATCCT GGAGA	TACCT GG			22
(2) INFORMATION	FOR SEQ ID NO:33:			
(A) LE (B) TY (C) SI	E CHARACTERISTICS: INGTH: 51 base pairs PE: nucleic acid TRANDEDNESS: double DPOLOGY: circular			
	LE TYPE: DNA SCRIPTION: pPIC.9 c	loning site		
(iii) HYPOTHE	TICAL: NO			
(iv) ANTI-SE	NSE: NO			
(xi) SEQUENC	CE DESCRIPTION: SEQ	ID NO:33:		
CTCGAGAAAA GAGAG	KICTGA AGCTTACGTA GA	ATTCCCTA GGCCGGCCGG	G	51
(2) INFORMATION	FOR SEQ ID NO:34:			
(A) LE (B) TY (C) SY (D) TO	TE CHARACTERISTICS: ENGTH: 40 base pairs (PE: nucleic acid munDeDNESS: single OPOLOGY: linear			
(11) MULECUI (A) PO	LE TYPE: DNA (primer TR 5' primer for amp) lifying human ob cDN	A sequence	

(iii) HYPOTHETICAL: NO

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4 C

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTATCTCTCG AGAAAAGAGT GCCCATCCAA AAAGTCCAAG

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (primer)
- (A) PCR 3' primer for amplifying human ob cDNA sequence
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCGCGAATTC TCAGCACCCA GGGCTGAGGT C

- (2) INFORMATION FOR SEO ID NO:36:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 - (A) PCR 5' primer for amplifying murine ob cDNA sequence
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTATCTCTCG AGAAAAGAGT GCCTATCCAG AAAGTCCAGG

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```
(2) INFORMATION FOR SEO ID NO:37:
      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 31 base pairs
           (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (primer)
          (A) PCR 3' primer for amplifying murine ob cDNA sequence
    (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: YES
    (xi) SEQUENCE DESCRIPTION: SEO ID NO:37:
GCGCGAATTC TCAGCATTCA GGGCTAACAT C
                                                                  31
(2) INFORMATION FOR SEO ID NO:38:
       (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 4 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: protein
             (A) DESCRIPTION: tetrapeptide at N-terminus of renatured murine
                 ob protein after thrombin cleavage
      (vi) ORIGINAL SOURCE:
             (A) ORGANISM: Murine
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:
Gly Ser His Met
(2) INFORMATION FOR SEQ ID NO:39:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 19 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY! linear
```

(A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1734

(ii) MOLECULE TYPE: DNA (primer)

(111) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO (vi) ORIGINAL SOURCE: (A) ORGANISM: Human (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39: CAAGACAAAT GAGATAAGG 19 (2) INFORMATION FOR SEQ ID NO:40: (i) SECUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1734 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (vi) ORIGINAL SOURCE: (A) ORGANISM: Human (xi) SECUENCE DESCRIPTION: SEC ID NO:40: AGAGTTACAG CTTTACAG 18 (2) INFORMATION FOR SEQ ID NO:41: (i) SECUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: Bingle (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS494 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (V1) ORIGINAL SOURCE: (A) ORGANISM: Human

CTAAACACCT TTCCATTCC 19

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

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(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear

```
(2) INFORMATION FOR SEC ID NO: 42:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 22 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (primer)
          (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS494
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (vi) ORIGINAL SOURCE:
          (A) ORGANISM: Human
    (xi) SECUENCE DESCRIPTION: SEC ID NO:42:
TTATATTCAC TITTCCCCTC TC
                                                                            22
(2) INFORMATION FOR SEQ ID NO:43:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (primer)
          (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS883
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (vi) ORIGINAL SOURCE:
          (A) ORGANISM: Human
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:
TGCAGTAAGC TGTGATTGAG
                                                                        20
(2) INFORMATION FOR SEQ ID NO:44:
     (i) SEQUENCE CHARACTERISTICS.
          (A) LENGTH: 20 base pairs
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(i1)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer	sWSS883
(iii)	HYPOTHETICAL: NO	
(1V)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:44:	
GTGCAGCT	TT AATTGTGAGC	20
(2) INFO	RMATION FOR SEQ ID NO:45:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer	sWSS2359
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:45:	
AGTGTTGTG	et treteere	18
(2) INFOR	RMATION FOR SEQ ID NO:46:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(i1)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer	6 W S S 2359
(111)	HYPOTHETICAL: NO	

(vi) ORIGINAL SOURCE: (A) ORGANISM: Human

(1v) ANTI-SENSE: NO

(2)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

AAAGGGGATG TGATAAGTG

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- (2) INFORMATION FOR SEQ ID NO:47:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 - (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2336
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GGTGTTACGT TTAGTTAC

18

- (2) INFORMATION FOR SEQ ID NO:48:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)(A) DESCRIPTION: sequence tagged-site specific PCR primer swss2336
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

195	
(2) INFORMATION FOR SEQ ID NO:49:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific	PCR primer sWSS1218
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE: (A) ORGANISM: Human	
(x1) SEQUENCE DESCRIPTION: SEQ ID NO:49:	
GCTCAACTGA CAGAAAAC	18
(2) INFORMATION FOR SEQ ID NO:50:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (primer)(A) DESCRIPTION: sequence tagged-site specifi	c PCR primer sWSS1218
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:	
GACTATGTAA AAGAAATGCC	20
(2) INFORMATION FOR SEQ ID NO:51:	
(1) SEQUENCE CHARACTERISTICS:(A) LENGTH: 18 base pairs	

(B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1402

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- (iii) HYPOTHETICAL: NO
- (1V) ANTI-SENSE: NO

AAAGGGCTTC TAATCTAC

- (vi) ORIGINAL SOURCE: (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

- (2) INFORMATION FOR SEQ ID NO:52:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1402
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CCTTCCAACT TCTTTGAC

- (2) INFORMATION FOR SEO ID NO:53:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

 - (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS999
 - (iii) HYPOTHELLCAL: NO
 - (iv) ANTI-SENSE: NO
 - (V1) ORIGINAL SOURCE: (A) ORGANISM: Human

197

(xi) SEQUENCE DESCRIPTION: SEO ID NO:53:

TAAACCCCCT TICTGTTC

1.8

- (2) INFORMATION FOR SEQ ID NO:54:
 - (i) SECURNCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 - (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS999
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TTGCATAATA GTCACACCC

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- (2) INFORMATION FOR SEQ ID NO:55:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)(A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1751
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:55

CCAAAATCAG AATTGTCAGA AG

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(2) INFORMATION FOR SEQ ID NO:56:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DRA (primer)(A) DESCRIPTION: sequence tagged-site specific PCR primer s	WSS1751
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:	
AAACCGAAGT TCAGATACAG	20
(2) INFORMATION FOR SEQ ID NO:57:	
(i) SEQUENCE CHARACTERISTICS: (A) LENTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (primer)(A) DESCRIPTION: sequence tagged-site specific PCR primer s	WSS1174
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:	
AATATCTGAC ATTGGCAC 18	1
(2) INFORMATION FOR SEQ ID NO:58:	
(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	

(D) TOPOLOGY: linear

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	(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site	specific	PCR	primer	eWSS117
	(iii)	HYPOTHETICAL: NO				
	(iv)	ANTI-SENSE: NO				
	(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human				
	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:58:				
TTA	GACCTY	ga gaaaagag			:	18
(2)	INFO	RMATION FOR SEQ ID NO:59:				
	(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear				
	(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site	specific	PCR	primer	eWSS206
	(iii)	HYPOTHETICAL: NO				
	(iv)	ANTI-SENSE: NO				
	(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human				
	(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:59:				
GTT	GCACAI	AT ACAAAATCC				19
(2)	INFO	RMATION FOR SEQ ID NO:60:				
	(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear				
	(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site	specific	PCR	primer	sWSS2061
	(iii)	HYPOTHETICAL: NO				
	(1V)	ANTI-SENSE: NO				

(vi) ORIGINAL SOURCE: (A) ORGANISM: Human

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CTTCCATTAG TGTCTTATAG

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- (2) INFORMATION FOR SEQ ID NO:61:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single

 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 - (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2588
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ATCACTACAC ACCTAATC

18

- (2) INFORMATION FOR SEQ ID NO:62:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2588
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

16 CCATTCTACA TITCCACC

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(2)	INFORMATION	FOR	SEO	ID	NO:63:	

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: Bingle
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (primer)

(A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS808

- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SECURNCE DESCRIPTION: SEC ID NO:63:

GGCTGTGTGA GCAAGATCCT AGGA

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(2) INFORMATION FOR SEO ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (primer)(A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS808
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEO ID NO:64:

TTGCCAGGCA AAGAGGGCTG GAC

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(2) INFORMATION FOR SEQ ID NO:65:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer BWSS1392 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (vi) ORIGINAL SOURCE: (A) ORGANISM: Human (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65: 18 CTCAGGTATG TCTTTATC (2) INFORMATION FOR SEQ ID NO:66: (1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer swSS1392 (iii) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (vi) ORIGINAL SOURCE: (A) ORGANISM: Human (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66: 18 TGTCTCTGCA TTCTTTTC (2) INFORMATION FOR SEQ ID NO:67: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sw331146 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GACACATACA AACACAAG

18

- (2) INFORMATION FOR SEO ID NO:68:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single

 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1148
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SECURNCE DESCRIPTION: SEC ID NO:68:

ATTGAGTTGA GTGTAGTAG

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- (2) INFORMATION FOR SEQ ID NO:69:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

 - (ii) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1529
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (V1) ORIGINAL SOURCE: (A) ORGANISM: Human
 - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:69:

CAGGGATTTC TAATTGTC

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(2) INFORMATION FOR SEQ ID NO:70:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (primer)
          (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS1529
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (vi) ORIGINAL SOURCE:
          (A) ORGANISM: Human
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:
                                                                    18
AAAAGATGGA GGCTTTTG
(2) INFORMATION FOR SEQ ID NO:71:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: DNA (primer)
          (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2619
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (vi) ORIGINAL SOURCE:
           (A) ORGANISM: Human
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:
                                                                         21
 CGTTAAGGGA AGGAACTCTG G
 (2) INFORMATION FOR SEQ ID NO:72:
      (1) SEQUENCE CHARACTERISTICS:
```

(A) LENGTH: 20 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear

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(īi)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR prim	er sWSS2619
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(x i)	SEQUENCE DESCRIPTION: SEQ ID NO:72:	
TGGCTTAG	BAG GAGTCAGGGA	20
(2) INFO	ORMATION FOR SEQ ID NO:73:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR prime	er swss404
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:73:	
ACCAGGGT	CA ATACAAAG	18
(2) INFO	RMATION FOR SEQ ID NO:74:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: sequence tagged-site specific PCR prime	r sWSS404
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

TAATGUGUCC TUCTUGCC

(2) INFORMATION FOR SEC ID NO:75:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (primer)

 (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2367
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CAATCCTGGC TTCATTTG

- (2) INFORMATION FOR SEQ ID NO:76:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 (A) DESCRIPTION: sequence tagged-site specific PCR primer sWSS2367
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

AAGGTGGGTA GGATGCTA

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(2) IN	FORMATION FOR SEQ ID NO:77:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDENNESS: single (D) TOPOLOGY: linear	
(i	i) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker UT528	
(ii	i) HYPOTHETICAL: NO	
(i	v) ANTI-SENSE: NO	
(v	i) ORIGINAL SOURCE: (A) ORGANISM: Human	
(x	i) SEQUENCE DESCRIPTION: SEQ ID NO:77:	
TGCAGT	AAGC TGTGATTGAG	20
(2) IN	FORMATION FOR SEQ ID NO:78:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(i	 i) MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker UT528 	
(ii	i) HYPOTHETICAL: NO	
(i	v) ANTI-SENSE: NO	
(v	i) ORIGINAL SOURCE: (A) ORGANISM: Human	
(ж	i) SEQUENCE DESCRIPTION: SEQ ID NO:78:	
GTGCAG	CTTT AATTGTGAGC	20
(2) IN	FORMATION FOR SEQ ID NO:79:	
(i) SEQUENCE CHARACTERISTICS: (A) LENOTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	

(D) TOPOLOGY: linear

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(11)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFMa065zg9	
(111)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:79:	
AGCTTCAA	GA CTTTNAGCCT	2
(2) INFO	RMATION FOR SEQ ID NO:80:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFMa065zg9	
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(x i)	SEQUENCE DESCRIPTION: SEQ ID NO:80:	
GGTCAGCA	GC ACTGTGATT	19
(2) INFO	RMATION FOR SEQ ID NO:81:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFMal25whl	
(iii)	HYPOTHETICAL: NO	

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TCACCTTGAG ATTCCATCC

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- (2) INFORMATION FOR SEQ ID NO:82:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 (A) DESCRIPTION: Marker AFMa125whl
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:

 (A) ORGANISM: Human
 - (xi) SECUENCE DESCRIPTION: SEQ ID NO:82:

AACACCGTGG TCTTATCAAA

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- (2) INFORMATION FOR SEQ ID NO:83:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 (A) DESCRIPTION: Marker AFM309yf10
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CATCCAAGTT GGCAGTTTTT

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(2) INFOR	MATION FOR SEQ ID NO:84:	
(i) s	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDMESS: single (D) TOPOLOGY: linear	
(ii) 1	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFM309yf10	
(iii) l	HYPOTHETICAL: NO	
(iv) i	ANTI-SENSE: NO	
(vi) (ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:84:	
AGATGCTGA	a TTCCCAGACA	
(2) INFOR	MATION FOR SEQ ID NO:85:	
(i) .	SEQUENCE CHARACTERISTICS: (A) LENGTH: 16 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) :	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFM218xf10	
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:85:	
TGGGCAACA	IC AGCAAA	16
(2) INFOR	MATION FOR SEQ ID NO:86:	
(1)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs	

(B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear

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(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFM218xf10		
(iii)	HYPOTHETICAL: NO		
(iv)	ANTI-SENSE: NO		
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human		
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:86:		
TGCAGTTA	GT GCCAATGTCA		20
(2) INFO	RMATION FOR SEQ ID NO:87:		
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 16 base pairs (B) TYPE: nucleic acid (C) SYRANDEDNESS: single (D) TOPOLOGY: linear		
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFM206xc1		
(iii)	HYPOTHETICAL: NO		
(iv)	ANTI-SENSE: NO		
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human		
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:87:		
CCAGGCCAT	G TGGAAC	16	
(2) INFOR	MATION FOR SEQ ID NO:88:		
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear		
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFM206xcl		

(111) HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (vi) ORIGINAL SOURCE: (A) ORGANISM: Human

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(wi) SECTIONCE DESCRIPTION: SEQ ID NO:88:	125	CEOTTENCE	DESCRIPTION:	SEO	ID	NC:88:
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AGTTCTTGGC TTGCGTCAGT

20

- (2) INFORMATION FOR SEQ ID NO:89:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 - (A) DESCRIPTION: Marker AFM199xh12
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TCTGATTGCT GGCTGC

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- (2) INFORMATION FOR SEQ ID NO:90:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (primer)
 (A) DESCRIPTION: Marker AFM199xh12
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human
 - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GCGCGTGTGT ATGTGAG

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(C) STRANDEDNESS: single (D) TOPOLOGY: linear

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(2) INFO	RMATION FOR SEQ ID NO:91:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFMa345vc9	
(iii)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:91:	
AGCTCTTG	GC AAACTCACAT	20
(2) INFO	RMATION FOR SEQ ID NO:92:	
(i)	SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii)	MOLECULE TYPE: DNA (primer) (A) DESCRIPTION: Marker AFMa345wc9	
(ili)	HYPOTHETICAL: NO	
(iv)	ANTI-SENSE: NO	
(vi)	ORIGINAL SOURCE: (A) ORGANISM: Human	
(xi)	SEQUENCE DESCRIPTION: SEQ ID NO:92:	
GCCTAAGG	GA ATGAGACACA	20
(2) INFO	RMATION FOR SEQ ID NO:93:	
(i)	SEQUENCE CHARACTERISTICS. (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid	

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(ii)	MOLE	CULE	TYPE:	DNA	(prime	r)			
	(A)	DES	CRIPTIO	ON:	primer	for	mouse	Pax4	gene

- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: murine
- (xi) SECUENCE DESCRIPTION: SEO ID NO:93:

GGGAGCCTTG TCCTGGGTAC AAAG

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- (2) INFORMATION FOR SEO ID NO:94:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 491 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA (A) DESCRIPTION: Recombinant murine met ob
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: murine
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 41..478
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:
- TCTAGATTTG AGTTTTAACT TTTAGAAGGA GGAATAACAT ATG GTA CCG ATC CAG 55 Met Val Pro Ile Gln
- AAA GTT CAG GAC GAC ACC AAA ACC TTA ATT AAA ACG ATC GTT ACG CGT 103 Lys Val Gin Asp Asp Thr Lys Thr Leu Ile Lys Thr Ile Val Thr Arg 10 15
- ATC AAC GAC ATC AGT CAC ACC CAG TCG GTC TCC GCT AAA CAG CGT GTT 151 Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser Ala Lys Gln Arg Val 25

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	ACC GGT CTG GAC TTC ATC CCG GGT CTG CAC CCG ATC CTA AGC TTG TCC 15																	
	ACC Thr	GGT Gly	CTG Leu 40	GAC Asp	TTC Phe	ATC Ile	CCG Pro	GGT Gly 45	CTG Leu	CAC His	CCG Pro	ATC Ile	CTA Leu 50	AGC Ser	TTG Leu	TCC Ser		199
	AAA Lys	ATG Met 55	GAC Asp	CAG Gln	ACC Thr	CTG Leu	GCT Ala 60	GTA Val	TAC Tyr	CAG Gln	CAG Gln	GTG Val 65	TTA Leu	ACC Thr	TCC Ser	CTG Leu		247
	CCG Pro 70	TCC Ser	CAG Gln	AAC Asn	GTT Val	CTT Leu 75	CAG Gln	ATC Ile	GCT Ala	AAC Asn	GAC Asp 80	CTC Leu	GAG Glu	AAC Asn	CTT	CGC Arg 85		295
	GAC Asp	CTG Leu	CTG Leu	CAC His	CTG Leu 90	CTG Leu	GCA Ala	TTC Phe	TCC Ser	AAA Lys 95	TCC Ser	TGC Cys	TCC Ser	CTG Leu	CCG Pro 100	CAG Gln		343
	ACC Thr	TCA Ser	GGT Gly	CTT Leu 105	CAG Gln	AAA Lys	CCG Pro	GAA Glu	TCC Ser 110	CTG Leu	GAC Asp	GGG Gly	GTC Val	CTG Leu 115	GAA Glu	GCA Ala		391
	TCC Ser	CTG Leu	TAC Tyr 120	AGC Ser	ACC Thr	GAA Glu	GTT Val	GTT Val 125	GCT Ala	CTG Leu	TCC Ser	CGT Arg	CTG Leu 130	CAG Gln	GGT Gly	TCC Ser		439
							CAG Gln 140								TAA	rgga		488
	TCC																	491
(2) INFORMATION FOR SEQ ID NO:95:																		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 146 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear																		
		(1	.i) N				: pr			oinar	at mu	rine	met	do:	prot	ein		
		(>	i) S	EQUE	NCE	DESC	RIPT	ION:	SEÇ) ID	NO: 9	5 :						
	Met 1	Val	Pro	Ile	Gln 5	Lys	Val	Gln	Asp	Asp 10	Thr	Lys	Thr	Leu	Ile 15	Lys		
	Thr	Ile	Val	Thr 20	Arg	Ile	Asn	qaA	Ile 25	Ser	His	Thr	Gln	Ser 30	Val	Ser		

Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro 35 45 Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln

Va. 6	l Leu 5	Thr	Ser	Leu	Pro 70		Gln	Asn	Val	Leu 75		Ile	Ala	Asn	As p 80	
Le	ı Glu	Asn	Leu	Arg 85	Авр	Leu	Leu	His	Leu 90		Ala	Phe	Ser	Lув 95		
Суя	s Ser	Leu	Pro 100		Thr	Ser	Gly	Leu 105	Gln	Lys	Pro	Glu	Ser 110		Авр	
Gly	/ Val	Leu 115		Ala	Ser	Leu	Tyr 120		Thr	Glu	Val	Val 125		Leu	Ser	
Arg	130		Gly	Ser	Leu	Gln 135	Asp	Ile	Leu	Gln	Gln 140		Asp	Val	Ser	
Pro	Glu	Cys														
(2)	INF	ORMA	TION	FOR	SEQ	ID I	10:9	6 :								
	(i	(; (;	A) Li B) T C) S	ENGT YPE : I'RANI	HARAC H: 45 nucl DEDNI	64 be leic SSS:	acio doul	pair:	5							
	(ii) MO			YPE:		ī: Re	ecom	oinaı	nt hi	uman	met	ob			
	(iii	HY	РОТНІ	TIC	AL: N	40										
	(iv	AN (rı-sı	NSE	NO.											
	(vi				OURCE (SM:		ın									
	(ix	(2		ME/I	CEY:		44									
	(xi	SE(QUENC	E DI	SCRI	PTIC	N: 5	EQ I	D NO	96:						
CAT	ATG Met							CAG Gln								4
	ACC Thr															3
	TCT Ser															14

										21/						
CCG	ATC	cTG	ACC	TTG	TCC	AAA	ATG	GAC	CAG	ACC	CTG	GCT	GTA	TAC	CAG	192
Pro	Ile	Leu	Thr	Leu	Ser	Lys	Met	Asp	Gln	Thr	Leu	Ala	Val	Tyr	Gln	
		50					55					60				
CAG	ATC	TTA	ACC	TCC	ATG	CCG	TCC	CGT	AAC	GTT	CIT	CAG	ATC	TCT	AAC	240
Gln	lle	Leu	Thr	Ser	Met		Ser	Arg	Asn	Val		Gln	Ile	Ser	Asn	
	65					70					75					
										~~~	~	CCR	dati.	TCC	מממ	288
GAC	CTC	GAG	AAC	CIT	CGC	GAC	CIG	CIG	CAC	370	tou	Ala	TTC	Ser	TAVE	
	Leu	Glu	Asn	Leu	Arg 85	АВР	Leu	Leu	птв	90	Lea	A10	Phe	-	95	
80					85					30						
<b>#</b> CC	TCC	CNC	CTC:	CCA	TOG	CCT	TCA	GGT	CTT	GAG	ACT	CTG	GAC	TCT	CTG	336
200	700	unc.	LAN	Pro	Trn	Ala	Ser	Glv	Leu	Glu	Thr	Leu	Asp	Ser	Leu	
261	СуБ	min	Deu	100		,,,,,		,	105					110		
GGC	GGG	GTC	CTG	GAA	GCA	TCC	GGT	TAC	AGC	ACC	GAA	GIT	GIT	GCT	CTG	384
Glv	Gly	Val	Leu	Glu	Ala	Ser	Gly	Tyr	Ser	Thr	Glu	Val	Val	Ala	Leu	
•			115					120					125			
																432
TCC	CGT	CTG	CAG	GGT	TCC	CTT	CAG	GAC	ATG	CTT	TGG	CAG	CTG	GAC	CIG	132
Ser	Arg		Gln	Gly	Ser	Leu		Asp	Met	Leu	Trp	Gin	Leu	Asp	Leu	
		130					135					140				
					~~~	TCC										454
				TAA	IGGA:	rec										
ser	145	Gly	Сув													
	145															
						TD :	TO - D	٠.								

- (2) INFORMATION FOR SEQ ID NO:97:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 147 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein (A) DESCRIPTION: Recombinant human met ob protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:
- Met Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys 5 1
- Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Gln Ser Val Ser 20
- Ser Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His Pro 40 35
- Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr Gln Gln 55
- Ile Leu Thr Ser Met Pro Ser Arg Asn Val Leu Gln Ile Ser Asn Asp 75 80 65 70

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Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser 85 90 95

Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly

Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser 115 120 125

Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser 130 135 140

Pro Gly Cys

- (2) INFORMATION FOR SEO ID NO:98:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (v) FRAGMENT TYPE: N-terminal His-tag
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

Met Gly Ser Ser His His His His His Ser Ser Gly Leu Val Pro

Arg Gly Ser His Met

- (2) INFORMATION FOR SEQ ID NO:99:
 - (i) SECUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (v) FRAGMENT TYPE: N-terminal His-tag
 - (xi) SEQUENCE DESCRIPTION. SEQ ID NO:99:

Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro

Arg Gly Ser Pro 20

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CLAIMS:

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- 1. An obesity (OB) polypeptide having about 145 to about 167 amino acids, capable of modulating body weight in an animal, or allelic variants or analogs, including fragments, thereof having the same biological activity.
- An OB polypeptide of claim 1, comprising the amino acid sequence of SEQ ID NOS: 2, 4, 5 or 6, or allelic variants or analogs, including fragments, thereof.
- An immunogenic fragment of an OB polypeptide according to claim 1
 - 4. An immunogenic fragment of an OB polypeptide selected from the group consisting of :
 - Val-Pro-Ile-Gin-Lys-Val-Gin-Asp-Asp-Thr-Lys-Thr-Leu-Ile-Lys-Thr (SEQ ID NO: 18):
 - Leu-His-Pro-lle-Leu-Ser-Leu-Ser-Lys-Met-Asp-Gln-Thr-Leu-Ala (SEQ ID NO: 19);
 - Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gin-Thr-Ser-Gly-Leu-Gin-Lys-Pro-Glu-Ser-Leu-Asp (SEO ID NO: 20); and
 - Ser-Arg-Leu-Gln-Gly-Ser-Leu-Gln-Asp-Ile-Leu-Gln-Gln-Leu-Asp-Val-Ser-Pro-Glu-Cvs (SEO ID NO: 21).
- A human OB polypeptide analog according to claim 2 wherein one or
 more amino acids selected from the group consisting of amino acids 53, 56, 71, 85,
 89, 92, 95, 98, 110, 118, 121, 122, 126, 127, 128, 129, 132, 139, 157, 159, 163,
 and 166 (according to the numbering of SEQ ID NO 4) is substituted with another

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- A human OB polypeptide analog according to claim 5 wherein substitution is with the divergent amino acid of the mouse OB polypeptide as set out in SEO ID NO: 2.
- 7. A human OB polypeptide analog according to claim 5 wherein substitution is with an alanine.
 - A human OB polypeptide analog according to claim 5 selected from the group consisting of polypeptides wherein:
 - (a) the serine residue at position 53 is substituted with glycine, alanine,
 valine, cysteine, methionine, or threonine;

- (b) the serine residue at position 98 is substituted with glycine, alanine, valine, cysteine, methionine, or threonine; and
- (c) the arginine residue at position number 92 is substituted with asparagine, lysine, histidine, glutamine, glutamic acid, aspartic acid, serine, threonine, methionine, or cysteine.
- An OB polypeptide analog according to claim 2 having 83 percent or greater amino acid sequence homology to the human OB polypeptide amino acid 20 sequence set out in SEQ ID NOS: 2, 4, 5 or 6.

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- 10. A human OB polypeptide analog according to claim 2 selected from the group consisting of polypeptides wherein:
 - (a) one or more aspartic acid residues is substituted with glutamic acid;
 - (b) one or more isoleucine residues is substituted with leucine;
 - (c) one or more glycine or valine residues is substituted with alanine;
 - (d) one or more arginine residues is substituted with histidine;
- (e) one or more tyrosine or phenylalanine residues is substituted with tryptophan;
- (f) one or more of residues 121 through 128 (according to the numbering 10 of SEO ID No:4) is substituted with glycine or alanine; and
 - (g) one or more residues at positions 54 through 60 or 118 through 166 (according to the number of SEQ ID NO: 4) is substituted with lysine, glutamic acid, cysteine, or proline.
- 15 11. An OB polypeptide according to any of claims 1, 2, 3, 5, 6 or 9 selected from the group consisting of polypeptides:
 - (a) having residues 1 through 21 deleted; and

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(b) polypeptides of subpart (a) having a methione at position 21, or having a glycine-serine-histidine-methionine sequence SEQ ID NO: 38 at positions 18 through 21, or having a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98) at positions 1 through

- 12. An OB polypeptide according to any of claims 1, 2, 3, 5, 6 or 9 selected from the group consisting of polypeptides:
 - (a) having residues 1 through 21 deleted; and
- (b) polypeptides of subpart (a) having a leucine-glutamic acid-lysine-arginine-squamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 26) at positions 14 through 21, or having a glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 27) at positions 18 through 21, or having a leucine-glutamic acid-lysine-arginine sequence (SEQ ID NO: 28) at positions 18 through 21, or having a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-serine-glycine-serine-proline sequence (SEQ ID NO: 99) at positions 2 through 21, or having a glycine-serine-proline sequence at positions 18 through 21.
- 13. An OB polypeptide according to any of claims 7, 8, 9, or 10 selected
 15 from the group consisting of polypeptides:
 - (a) having residues 1 through 21 deleted; and
- (b) polypeptides of subpart (a) having a methione at position 21, or having a glycine-serine-histidine-methionine sequence (SEQ ID NO: 38) at positions 18 through 21, or having a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-estrine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98) at positions 1 through 21, or having a leucine-glutamic acid-lysine-arginine-glutamic acid-alanine sequence (SEQ ID NO: 26) at positions 14 through 21, or having a glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 27) at positions 18 through 21, or having a leucine-glutamic acid-lysine-arginine sequence (SEQ ID NO: 28) at positions 18 through 21, or having a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-serine-glycine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-proline sequence (SEQ ID NO: 99) at positions 2 through 21, or having a glycine-serine-proline sequence at positions 18 through 21.

- 14. A human OB polypeptide truncated analog according to claim 2 selected from the group (according to the numbering of SEQ ID NO: 4) consisting of polypeptides wherein:
 - (a) one or more residues at positions 121 to 128 are deleted;
- 5 (b) residues 1-116 are deleted;
 - (c) residues 1-21 and 54 to 167 are deleted;
 - (d) residues 1-60 and 117 to 167 are deleted;
 - (e) residues 1-60 are deleted;
 - (f) resides 1-53 are deleted;
- 10 (g) an analog of subpart (a) wherein residues 1-21 are deleted: and
 - (h) an analog of subpart (a) through (g) having an N-terminal amino acid or amino acid sequence selected from the group consisting of:
 - (1) methionine,
 - (2) a glycine-serine-histidine-methionine sequence (SEQ ID NO:
- 15 38),

- (3) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98),
- (4) a leucine-glutamic acid-lysine-arginine-glutamic acid-alanineglutamic acid-alanine sequence (SEQ ID NO: 26),
- (5) a glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 27).
 - (6) a leucine-glutamic acid-lysine-arginnine sequence (SEQ ID NO:
 28).
- 25 (7) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-proline sequence (SEQ ID NO: 99), and
 - (8) a glycine-serine-proline sequence.
- 30 15. A recombinant OB polypeptide according to any of claims 1 through 14.

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- A chemically synthesized OB polypeptide according to any of claims
 through 14.
- 17. A derivative of an OB polypeptide according to any of claims 1 through
 16 having one or more chemical moieties attached thereto.
 - A derivative of claim 17, wherein the chemical moiety is a watersoluble polymer.
- 10 19. A derivative of claim 18, wherein the water-soluble polymer is polyethylene glycol.
 - A derivative of claim 19 which is mono-, di-, tri- or tetrapegylated.
- 15 21. A derivative of claim 20 which is N-terminal monopegylated.
 - A derivative of claim 21 which is an OB polypeptide comprising the amino acid residues 22 through 167 of SEQ ID NO:4 or residues 22 through 166 of SEO ID NO: 6.

- 23. A derivative of claim 21 which is an OB polypeptide comprising the amino acid sequence of residues 22 through 167 of SEQ ID NO: 4 or residues 22 through 166 of SEQ ID NO: 6 and having a methionine at position 21.
- 25 24. An isolated nucleic acid molecule encoding an OB polypeptide according to any of claims 1 through 6, 9 or 11.
 - 25. An isolated nucleic acid molecule encoding an OB polypeptide according to any of claims 7, 8, 10, 12, 13 or 14.

- 26. A DNA molecule for use in securing expression of an OB polypeptide having the biological activity of modulating body weight in a mammal, the DNA being selected from the group consisting of:
- (a) the DNA molecules set out in SEQ ID NOS: 1 and 3 or fragments 5 thereof.
 - (b) DNA molecules which hybridize to the DNA molecules defined in (a) or hybridizable fragments thereof; and
 - (c) DNA molecules that code on expression for the amino acid sequence encoded by any of the foregoing DNA molecules.

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- 27. A DNA molecule according to claim 26 which is the human genomic DNA molecule of SEQ ID NOS: 22 and 24.
- 28. A DNA molecule according to claim 24 encoding a polypeptide having an amino acid sequence selected from the group consisting of the amino acid 15 sequences set out in:
 - (a) SEQ ID NO: 2;
 - (b) amino acids 22 through 167 of SEQ ID NO: 2;
 - (c) SEQ ID NO: 4;
 - (d) amino acids 22 through 167 of SEQ ID NO: 4;
- 20 (e) SEQ ID NO: 5;
 - (f) amino acids 22 through 166 of SEQ ID NO: 5;
 - (g) SEQ ID NO: 6;
 - (h) amino acid 22 through 166 of SEQ ID NO: 6; and
 - (i) the amino acid sequences of subpart (b) (d), (f) or (h) having an N-
- 25 terminal amino acid or amino acid sequence selected from the group consisting of:
 - (1) methionine,
 - a glycine-serine-histidine-methione sequence (SEQ ID NO: 38),

and

(3) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98).

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29. A DNA molecule according to claim 28 encoding an amino acid of subpart (b), (d), (f) or (h) having an N-terminal amino acid sequence selected from the group consisting of:

- a leucine-glutamic acid-lysine-arginine-glutamic acid-alanineglutamic acid-alanine sequence (SEQ ID NO: 26),
 - (2) a glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 27),
 - ${\rm (3)} \qquad {\rm a \ leucine\ -glutamic\ acid\ -ly sine\ -arginine\ sequence\ (SEQ\ ID\ NO:\ 28)},$
 - (4) a methionine-glycine-serine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-proline sequence (SEQ ID NO: 99), and
 - (5) a glycine-serine-proline sequence.
- 15 30. A DNA molecule according to claim 24 comprising the sequence set out as the protein coding sequence of SEQ ID NO: 3.
 - 31. A DNA molecule according to claim 24 comprising the sequence set out as the sequence encoding amino acids 22 through 167 of SEQ ID NO: 3.
 - A detectably labeled nucleic acid molecule hybridizable to a DNA molecule according to any one of claims 24 to 31.
- 33. A nucleic acid hybridizable to a non-coding region of an OB nucleic coding region is selected from the group consisting of an intron, a 5 non-coding region, and a 3 non-coding region.
 - An oligonucleotide primer for amplifying human genomic DNA encoding an OB polypeptide.

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35. An oligonucleotide according to claim 32, which is selected from the group consisting of:

HOB 1gF 5'-CCCAAGAAGCCCATCCTG-3' (SEQ ID NO. 29); HOB 1gR 5'-GACTATCTGGGTCCAGTGCC-3' (SEQ ID NO. 30); HOB 2gF 5'-CCACATGCTGAGCACTTGTT-3' (SEQ ID NO. 31); and HOB 2gR 5'-CTTCAATCCTGGAGATACCTGG-3' (SEO ID NO. 32).

- A vector which comprises a DNA molecule according to any of claims
 through 31.
- 37. An expression vector which comprises a DNA molecule according to any of claims 24, 26, 30 or 31 operatively associated with an expression control sequence.
- 15 38. An expression vector which comprises a DNA molecule according to claim 27 or 29 operatively associated with an expression control sequence.
 - 39. An expression vector which comprises a DNA molecule according to claim 25 operatively associated with an expression control sequence.

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- 40. An unicellular host transformed or transfected with a DNA molecule of claim 24 or 25 or an expression vector of any one of claims 36 through 39.
- 41. A unicellular host according to claim 40, wherein the unicellular host is selected from the group consisting of bacteria, yeast, mammalian cells, plant cells, insect cells, and human cells in tissue culture.
- 42 The unicellular host of claim 40, wherein the unicellular host is selected from the group consisting of E. coli, Pseudomonas, Bacillus, Streptomyces, 30 yeast, CHO, R1.1, B-W, LM, COS 1, COS 7, BSC1, BSC40, BMT10, and Sf9 cells.

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43. A unicellular host according to claim 40 wherein the unicellular host is a yeast host selected from the group consisting of Saccharomyces, Pichia, Candida, Hansenula and Torulopsis.

- 44. A mammalian cell containing an OB polypeptide encoding DNA sequence and modified in vitro to permit higher expression of OB polypeptide by means of a homologous recombinational event consisting of inserting an expression regulatory sequence in functional proximity to the OB polypeptide encoding sequence.
- 45. A cell according to claim 44 wherein the expression regulatory sequence is an OB polypeptide expression regulatory sequence and the homologous recombinational event replaces a mutant OB polypeptide expression regulatory sequence.
- 15 46. A cell according to claim 45 wherein the expression regulatory sequence insert is not an OB polypeptide regulatory sequence.
 - 47. A method for preparing an OB polypeptide comprising:
- (a) culturing a cell according to any of claims 40 through 46 under 20 conditions that provide for expression of the OB polypeptide; and
 - (b) recovering the expressed OB polypeptide.
 - 48. The method according to claim 47 wherein the cell is a bacterium or a yeast.

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- 49. The method according to claim 47 or 48 further comprising:
- (c) chromatographing the OB polypeptide on a Ni-chelation column; and
- (d) purifying the OB polypeptide by gel filtration.

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50. The method according to claim 49, further comprising after step (c) and before step (d) chromatographing the OB polypeptide on a strong cation exchanger column.

- 5 51. An antibody specific for an OB polypeptide according to any of claims 1 through 16 or produced by the method of claims 47 through 50.
 - An antibody according to claim 51 which is a monoclonal or polyclonal antibody.

53. An antibody according to claim 52 labeled with a detectable label.

 An immortal cell line that produces a monoclonal antibody according to claim 52.

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- 55. A method for preparing an antibody specific to an OB polypeptide, comprising:
- (a) conjugating an OB polypeptide according to any of claim 1 through 16 or produced by the method of claims 47 through 50 to a carrier protein;
- 20 (b) immunizing a host animal with the OB polypeptide fragment-carrier protein conjugate of step (a) admixed with an adjuvant; and
 - (c) obtaining antibody from the immunized host animal.
- 56. A method for measuring the presence of an OB polypeptide in a 25 sample, comprising:
 - (a) contacting a sample suspected of containing an OB polypeptide with an antibody that specifically binds to the OB polypeptide under conditions which allow for the formation of reaction complexes comprising the antibody and the OB polypeptide; and
- 30 (b) detecting the formation of reaction complexes comprising the antibody and OB polypeptide in the sample,

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wherein detection of the formation of reaction complexes indicates the presence of OB polypeptide in the sample.

- 57. The method of claim 56 in which the antibody is bound to a solid phase5 support.
 - 58. An in vitro method for evaluating the level of OB polypeptide in a biological sample comprising:
- (a) detecting the formation of reaction complexes in a biological sample
 10 according to the method of claim 56 or 57; and
 - (b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of OB polypeptide in the biological sample.
- 15 59. An in vitro method for detecting or diagnosing the presence of a disease associated with elevated or decreased levels of OB polypeptide in a mammalian subject comprising:
 - (a) evaluating the level of OB polypeptide in a biological sample from a mammalian subject according to claim 58; and
- (b) comparing the level detected in step (a) to a level of OB polypeptide present in normal subjects or in the subject at an earlier time, wherein an increase in the level of OB polypeptide as compared to normal levels indicates a disease associated with elevated levels of OB polypeptide, and decreased level of OB polypeptide as compared to normal levels indicates a disease associated with decreased levels of OB polypeptide.
 - 60. An in vitro method for monitoring a therapeutic treatment of a disease associated with elevated or decreased levels of OB polypeptide in a mammalian subject comprising evaluating the levels of OB polypeptide in a series of biological samples obtained at different time points from a mammalian subject undergoing a

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therapeutic treatment for a disease associated with elevated or decreased levels of OB polypeptide according to the method of claim 58.

- 61. A pharmaceutical composition comprising an OB polypeptide according
 to any of claims 1 through 16 or produced by the process of claims 47 through 50 and a pharmaceutically acceptable carrier.
 - A pharmaceutical composition of claim 61 for reducing the body weight of an animal.
 - 63. A pharmaceutical composition for increasing the body weight of an animal comprising an antagonist of an OB polypeptide according to any of claims 1 through 16 or produced by the process of claims 47 through 50 and a pharmaceutically acceptable carrier.

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- 64. The pharmaceutical composition of claim 63, wherein the antagonist is selected from the group consisting of an antibody that binds to and neutralizes the activity of the OB polypeptide, a fragment of the OB polypeptide that binds to but does not activate the OB polypeptide receptor, and a small molecule antagonist of the OB polypeptide.
- 65. A body appearance improving cosmetic composition for reducing the body weight of an individual comprising an OB polypeptide according to any of claims 1 through 16 or produced by the process of claims 47 through 50 and an acceptable carrier.
- 66. A body appearance improving cosmetic composition for increasing the body weight of an individual comprising an antagonist of an OB polypeptide according to any of claims 1 through 16 or produced by the process of claims 47 through 50 and an acceptable carrier.

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67. A cosmetic composition according to claim 66, wherein the antagonist is selected from the group consisting of an antibody that binds to and neutralizes the activity of the OB polypeptide, a fragment of the OB polypeptide that binds to but does not activate the OB polypeptide receptor, and a small molecule antagonist of the OB polypeptide.

68. A cosmetic process for improving the body appearance of an individual, wherein a cosmetic composition according to any of claims 64 through 67 is administered to the individual in a dose amount sufficient to modulate the individual's body weight to a desired level.

- 69. Use of an antisense nucleic acid molecule hybridizable to a nucleic acid encoding an OB polypeptide according to any of claims 1 through 4 or 9 for manufacture of a medicament for modifying the body weight of a mammal.
- 10 70. Use of a nucleic acid molecule encoding an OB polypeptide according to any of claims 1 through 16 or produced by the process of claims 47 through 50 for the manufacture of a gene therapy medicament for modifying the body weight of an animal
- 15 71. Use of an OB polypeptide according to any of the claims 1 through 16 or produced by the method of claims 47 through 50 for the manufacture of a medicament for modification of the body weight of an animal.
- 72. Use of an OB polypeptide according to any of the claims 1 through 16 or produced by the method of claims 47 through 50 for the manufacture of a medicament for modification of the body weight of a mammal in treating a disorder selected from the group consisting of diabetes, high blood pressure and high cholestern).
- 25 73. Use of an ob polypeptide according to any of the claims 1 through 16 or produced by the method of claims 47 through 50 for the manufacture of a medicament for modification of the body weight of a mammal for use in combination with a medicament for treating diabetes, high blood pressure and high cholesterol.

- 74. Use of an antagonist of an OB polypeptide according to any of claims 1 through 16 or produced by the process of claims 47 through 50 for the manufacture of a medicament for increasing the body weight of an animal.
- The use according to claim 74 wherein the antagonist is selected from the group consisting of an antibody that binds to and neutralizes the activity of the OB polypeptide, a fragment of the OB polypeptide that binds to but does not activate the OB receptor, and a small molecule antagonist of the OB polypeptide.
- 76. The use according to any of claims 71 through 75 for the manufacture of a medicament for intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, nasal, oral or pulmonary delivery.

61	121 22	181 42	241 62	301 82	361 102	421 122	481
GGAI L'C'CI GCTCCAGCAGGTGCAAGAGAAGAAGAAGATCCCAGGGAGGAAATGTG M_C	CTGGAGACCCCTGTGTCCTGTGGCTTTGGTCCTATCTGTTCAAGCAGT	GCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACCAGGAT P I Q K V Q D D T K T L I K T I V T R I	CAATGACATTTCACACGCAGTGGGTATCCGCCAAGCAGGGGTCACTGGCTTGGACTT N D I S H T $\underline{ extbf{o}}$ S V S A K $ extbf{o}$ R V T G L D F	CATTCCTGGGCTTCACCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCAGTCTA	TCAACAGGTCCTCACCAGCTGCCTTCCCAAATGTGCTGCAGATAGCCAATGACCTGGA	GAATCTCCGAGACCTCCTCCATCTGCCTTCTCCAAGAGCTGCTCCCTGCCTCAGAC	CAGTGGCCTGCAGAAGCCAGAGAGCTGGATGGCGTCCTGGAAGCCTCACTCTACTCCAC

NANGELIEGENTITERECRUECINGENEGEN PROPRIETE VALS R. L. G. G. S. L. Q. D. I. L. Q. L. D.	541 162
IGTTAGCCCTGAATGCTGAAGTTTCAAAGGCCACCAGGCTCCCAAGAATCATGTAGAGGG	601 167
\AGAA\CCTTGGCTTCCAGGGGTCTTCAGGAGAAGAGAGCCATGTGCACACATCCATC	661
CATTICTCTCCCTCCTGTAGACCACCCATCCAAAGGCATGACTCCACAATGCTTGACTC	721
AGTTATCCACACAACTTCATGAGCACAAGGAGGGGCCAGCCTGCAGAGGGGACTCTCAC	781
TAGTICTTCAGCAAGTAGAGATAAGAGCCATCCCATCCCCTCCATGTCCCACCTGCTCC	841
3GGTACATGTTCCTCCGTGGGTACACGCTTCGCTGCGGCCCAGGAGGAGGTGAGGGA	901
GGGTAGAGCCTTTGGGCTGTCTCAGAGTCTTTGGGAGCACCGTGAAGGCTGCATCCACA	961
:ACAGCTGGAAACTCCCAAGCAGCACGATGGAAGCACTTATTTAT	1021
TATTTIGGATGGATCTGAAGCAAGGCATCAGCTTTTTCAGGCTTTGGGGGTCAGCAGGA	1081
TGAGGAAGGCTCCTGGGGTGCTTTCAATCCTATTGATGGGTCTGCCCGAGGCAAACC 114	1147

1801 1861 1381 1441 1501 1561 1621 1681 1741 1261 1321 CACTTAGCAGATGGTCCTGAGCCCTGGGCCAGCACTGCTGAGGAAGTGCCAGGGCCCCAG TCTTANGCAGGTAGACGTTTGCATGCCAATATGTGGTTCTCATCTGATTGGTTCATCCAA TTTGTIGGCATATTGCTGAGCTCAGGGAGTGAGGGCCCCCACATTTGAGACAGTGAGCCCC AGTAGNACCCTGTCTCCCACCCATTCTGTGGGGAGTTTTGTTCCAGTGGGAATGAGAAAT TAATTITTGAGTGACTGGAAGGAAGGTTGGGATCTTCCAAACAAGAGTCTATGCAGGTAG GGCATITICITACCTCTGTGGCCACATAGTGTGGCTTTGTGAAAAGGACAAAGGAGTTGA CTCTTI CCGGAACATTTGGAGTGTACCAGGCACCCTTGGAGGGGCTAAAGCTACAGGCCT **AAGAAVAGGGTCCCTGGTGTAGATCTCCAAGGTTGTCCAGGGTTGATCTCACAATGCGTT** ACGTTTGCAGCGGCATTGCCGGGAGCATAGGCTAGGTTATTATCAAAAGCAGATGAATTT

FIG. 1C

2521 2581 2281 2401 2461 2101 2161 222.1 2341 1981 2041 GCATGAAAGACGTTTTTACAGCATGAACCTGTCTACTCATGCCAGCACTCAAAAACCTTG TGAGCTAGAGAAGCTCACCACATACATATAAAATCAGAGGCTCATGTCCCTGTGGTTAG TGCAGTCTATTGCATTTACATACCGCATTTCAGGGCACATTAGCATCCACTCCTATGGTA GCACACTGTTGACAATAGGACAAGGGATAGGGGTTGACTATCCCTTATCCAAAATGCTTG **GGACTAGAAGAGTTTTGGATTTTAGAGTCTTTTCAGGCATAGGTATATTTGAGTATATA AAAATGAGATATCTTGGGGATGGGGCCCAAGTATAAACATGAAGTTCATTTATATTTCAT AATACCGTATAGACACTGCTTGAAGTGTAGTTTTATACAGTGTTTTAAATAACGTTGTAT** GCCAGGCTGCCAGAATTGCCCTTCGGGCTGGAGGATGAACAAAGGGGCTTGGGTTTTTCC ATCACCCCTGCACCCTATGTCACCATCAAACTGGGGGGGCAGATCAGTGAGGAGGACACTTG GTCTTCAACAGGTGTGAAAGAACCTGAGCTGAGGGTGACAGTGCCCAGGGGAACCCTGCT **ACCCTACTCGCGGCGGTGTACTCCACCACAGCAGCACCGCACCGCTGGAAGTACAGTGCT**

FIG. 1D

FIG. 1E

2641 2707 2761 2821 TCCAGCCAGGTCATACCCTGTGGAGGTGAGCGGGATCAGGTTTTGTGGTGCTAAGAGAGG GGGTTTTGGAGCAGTTTGGATCTTGGGTTTTCTGTTAAGAGATGGTTAGCTTATACCTAA AACCAT AAT GGCAAACA GGCT GCA GGACCA GATCCT CA GCCCT GAAGT GT GCCCT **AGTTGGAGGTAGATTTTGGAGGATCTGAGGGC**

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FIG. 2A

ê 450 200 550 င္သ 20 8 220 8 320 8 GAAGCITCAG GCTACTCCAC AGAGGTGGTG GCCCTGAGCA GGCTGCAGGG CTCACCAGTA TGCCTTCCAG AAACGTGATC CAAATATCCA ACGACCTGGA TGCCCTGGGC CAGTGGCCTG GAGACCTTGG ACAGCCTGGG GGGTGTCCTG GTCTCTGCAG GACATGCTGT GGCAGCTGGA CCTCAGCCCT GGGTGCTGAG CTCCAAACAG AAAGTCACCG GTTTGGACTT CATTCCTGGG CTCCACCCCA TTGGGGAACC CTGTGCGGAT TCTTGTGGCT TTGGCCCTAT CTTTTCTATG TCCAAGCTGT GCCCATCCAA AAAGTCCAAG ATGACACCAA AACCCTCATC AAGACAATTG TCACCAGGAT CAATGACATT TCACACACGC AGTCAGTCTC ICCTGACCTT ATCCAAGATG GACCAGACAC TGGCAGTCTA CCAACAGATC GAACCTCCGG GATCTTCTTC ACGTGCTGGC CTTCTCTAAG AGCTGCCACT ---G--GTTG CAAGGCCCAA GAAGCCCA-- -TCCTGGGAA GGAAAATGCA

902 909 650 GCCTTGAAGG TCACTCTTCC TGCAAGGACT -ACGTTAAGG GAAGGAACTC TGGTTTCCAG GTATCTCCAG GATTGAAGAG CATTGCATGG ACACCCCTTA TCCAGGACTC TGTCAATTTC CCTGACTCCT CTAAGCCACT CTTCCAAAGG

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SER ARG ASN VAL ILE GLN ILE SER ASN ASP LEU GLU ASN LEU ARG ASP LEU LEU HIS VAL LEU ALA PHE SER LYS SER CYS HIS LEU PRO TRP ALA SER GLY LEU GLU THR LEU ASP SER LEU GLY GLY VAL LEU THR LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE SER HIS THR GLN SER VAL SER SER LYS GLN LYS VAL THR GLY LEU ASP GLN THR LEU ALA VAL TYR GLN GLN ILE LEU THR SER MET PRO GLU ALA SER GLY TYR SER THR GLU VAL VAL ALA LEU SER ARG LEU GLN GLY SER LEU GLN ASP MET LEU TRP GLN LEU ASP LEU SER PRO LEU PHE TYR VAL GLN ALAIVAL PRO ILE GLN LYS VAL GLN ASP ASP ASP PHE ILE PRO GLY LEU HIS PRO ILE LEU THR LEU SER LYS MET GLY CYS END

106

121

136

151

166

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr

46

61

SHESTITHTE SHEET (RULE 26)

16

9/67

20 100 120 167 MCWRPLCRFL WLWSYLSYVQ AVPIQKYQDD TKTLIKTIVT RINDISHTQS VSAKORVTGL DFIPGLHPIL SLSKMDOTLA VYQOVLTSLP SONVLOIAND VSSKOKYTGL DFIPGLHPIL TLSKMDOTLA VYQQILTSMP SRNVIQISND LENLRDLLHL LAFSKSCSLP QTSGLQKPES LDGVLEASLY STEVVALSRL LENLRDLLHV LAFSKSCHLP WASGLETLDS LGGVLEASGY STEVVALSRL OGSLADILAA LDVSPEC OGSLODMLWO LDLSPGC Mouse HUMAN HUMAN Mouse HUMAN HUMAN Mouse Mouse

MET CYS TRP ARG PRO LEU CYS ARG PHE LEU TRP LEU TRP SER TYR

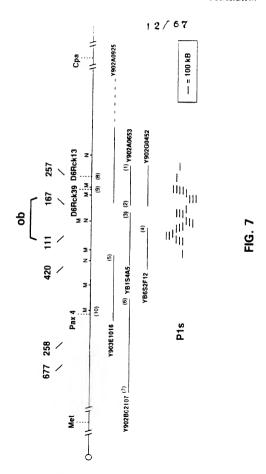
10/67												
ILE	Asp	Asp	SER	Asp	GLN	GLU	GLN	GLU				
Asp	LEO		PRO	ARG	PRO	LEO	LEO	PRO				
ASN	GLY	Lys			LEU		ARG					
ILE	THR	SER	SER	ASN	SER		Ser	YAL				
ARG	\AL	LEU	THE	GĽU	C۲s	Asp	LEU	Asp				
Ŧ	ARG	SER	LEG	LEU	SER	LEU	Ā	LEG				
VAL	GLN	LEU	VAL	Asp	Lys	SER	VAL	GLN				
ILE	Lys			ASN	SER	GLU	VAL	GLN				
TH H	Ā	PRO	GLN	Ą	PHE	PRO	GLU	LEU				
Lys	SER	HIS	TYR	ILE	Ą	Lys	THR	Ī				
ILE	VAL	LEG	VAL	GLN	LEU	GLN	SER	Asp				
	SER	GLY	¥.	LEU	LEU	Leu	TYR	<u>5</u>				
THR	THR	PRO	LEO	VAL	HIS	GLY	LEU					
Lys	HIS	ILE	TH	ASN	LEU	SER	SER	SER	Ex			
THR	SER	PHE	GLN	GLN	LEU	TH	Ą	GLY	CYS			
	ASN	Lys Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp His Thr Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASPHIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET ASP THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET ASP THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP LEU HIS LEU LEU ALA PHE SER LYS SER CYS SER LEU PRO GLN	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET ASP THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP LEU HIS LEU LEU ALA PHE SER LYS SER CYS SER LEU PRO GLN SER GLY LEU GLN LYS PRO GLU SER LEU ASP GLY VAL LEU GLU	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET ASP THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP LEU HIS LEU LEU ALA PHE SER LYS SER CYS SER LEU PRO GLN SER GLY LEU GLN LYS PRO GLU SER LEU ASP GLY VAL LEU GLU SER LEU TYR SER THR GLU VAL VAL ALA LEU SER ARG LEU GLN	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU SER LYS MET ASP THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP LEU HIS LEU LEU ALA PHE SER LYS SER CYS SER LEU PRO GLN SER GLY LEU GLN LYS PRO GLU SER LEU ASP GLY VAL LEU GLU SER LEU TYR SER THR GLU VAL VAL ALA LEU SER ARG LEU GLN SER LEU GLN ASP ILE LEU GLN GLN LEU ASP VAL SER PRO GLU	LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE HIS THR SER VAL SER ALA LYS GLN ARG VAL THR GLY LEU ASP ILE PRO GLY LEU HIS PRO ILE LEU SER LEU PRO SER THR LEU ALA VAL TYR GLN GLN VAL LEU THR SER LEU PRO SER ASN VAL LEU GLN ILE ALA ASN ASP LEU GLU ASN LEU ARG ASP LEU HIS LEU LEU ALA PHE SER LYS SER CYS SER LEU PRO GLN SER GLY LEU GLN LYS PRO GLU SER LEU ASP GLY VAL LEU GLU SER LEU TYR SER THR GLU VAL VAL ALA LEU SER ARG LEU GLN SER LEU GLN ASP ILE LEU GLN GLN LEU ASP VAL SER PRO GLU		

 MET HIS TRP GLY THR LEU CYS GLY PHE LEU TRP LEU TRP PRO TYR

FIG. 6

ξζ LEU PHE TYR VAL GLN ALA VAL PRO ILE GLN LYS VAL GLN ASP ASP THR LYS THR LEU ILE LYS THR ILE VAL THR ARG ILE ASN ASP ILE SER HIS THR SER VAL SER SER LYS GLN LYS VAL THR GLY LEU ASP PHE ILE PRO GLY LEU HIS PRO ILE LEU THR LEU SER LYS MET ASP GLN THR LEU ALA VAL TYR GLN GLN ILE LEU THR SER MET PRO SER ARG ASN VAL ILE GLN ILE SER ASN ASP LEU GLU ASN LEU ARG ASP LEU LEU HIS VAL LEU ALA PHE SER LYS SER CYS HIS LEU PRO TRP ALA SER GLY LEU GLU THR LEU ASP SER LEU GLY GLY VAL LEU GLU ALA SER GLY TYR SER THR GLU VAL VAL ALA LEU SER ARG LEU GLN GLY SER LEU GLN ASP MET LEU TRP GLN LEU ASP LEU SER PRO 31 46 9/ 106 136 151 166 61 121

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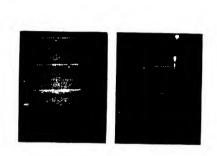


FIG. 8

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FIG. 9

FIG. 10

+10 +20 +30 +40 cagecãega AAATGTGCT GAGACCCCTG CACGITCITC TICTICIAGE GICCCGICCI TITACACGAC CICTGGGGAC +10 +20 +30 +40
TGTCGGGTCC NGTGGNTTTG GTCCTATCTG TCTTATGTNC AAGCAGTGCC ACAGCCCAGG NCACCNAAAC CAGGATAGAC AGAATACANG TTCGTCACGG 2---3----

ATAGGTCTTT CAGGTCCTAC TGTGGTTTTC GGAGTAGTTC TGGTAACAGT +30 +10 +20 NCAGGATCAC TGANATTTCA CACACG

+10 +20 +30 +40
TATCCAGAAA GTCCAGGATG ACACCAAAAG CCTCATCAAG ACCATTGTCA

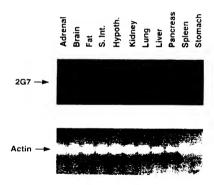


FIG. 11A

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white fat brain small intestine stomach pancreas lung testis heart spleen liver

28S -

18S — '

10 11 7

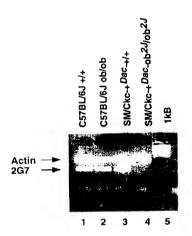


FIG. 12A

2G7

Actin

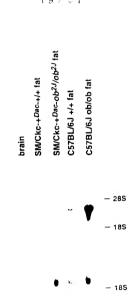


FIG. 12B

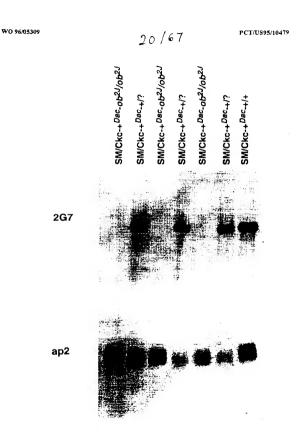
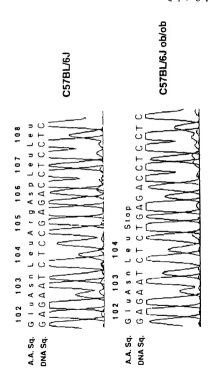


FIG. 13





<u>i</u>G. 14

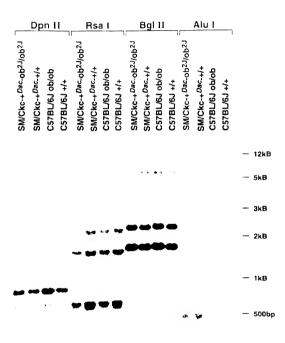


FIG. 15A

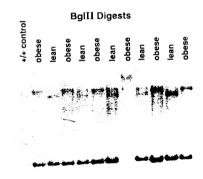


FIG. 15B



FIG. 16

17 PROMOTER PRIMER 69348-1

^----

<u>BGLII</u> AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTACA 17 PROMOTER

MetGLYSERSERHISHISHISHISHISSERSERGLY

<u>LEUVALPROARGGLYSER</u>HISMETLEUGLUASPPROALAALAASNLYSALAARGLYSGLUALAGLULEUALA NDET XHOT BAMHI CTGGTGCCGCGGCGCGTATGCTCGAGGATCCCGCTGCTACAAAGGAAAGGAAGCTGABTTGGCT THROMBIN

BPU11021 GCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG ALAALATHRALAGLUGLNEND

T7 TERMINATOR PRIMER #69337-1

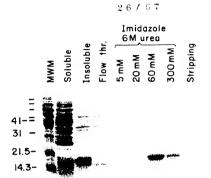


FIG. 18A



FIG. 18B

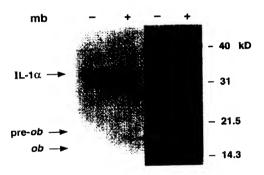


FIG. 19A

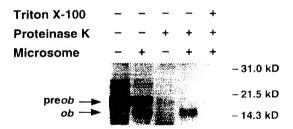


FIG. 19B

FIG. 20A

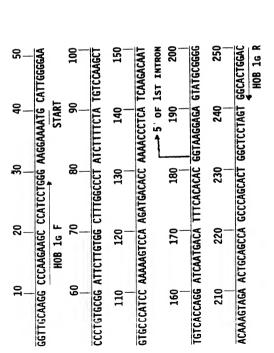
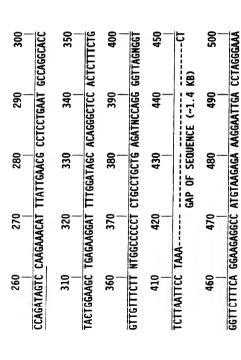


FIG. 20B



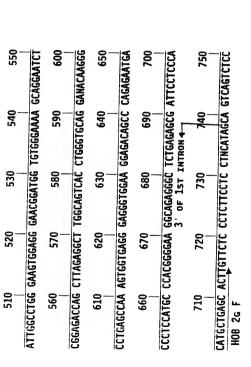


FIG. 20C

00° 00°	TCCACCCCAT	850	CAACAGATCC	006	CGACCTGGAG	950	GCTGCCACTT	1000	GGTGTCCTGG
790	ATTCCTGGGC	840	GGCAGTCTAC	890	AAATATCCAA	940	TTCTCTAAGA	066	CAGCCTGGGG
780	TTTGGACTTC	830	ACCAGACACT	880	AACGTGATCC	930	CGTGCTGGCC	086	AGACCTTGGA
770	CCAAACAGA AAGTCACCGG TTTGGACTTC ATTCCTGGGC TCCACCCAT	820	CTGACCTTA TCCAAGATGG ACCAGACACT GGCAGTCTAC CAACAGATCC	870	CACCAGTAT GCCTTCCAGA AACGTGATCC AAATATCCAA GGACCTGGAG	920	ACCTCCGGG ATCTTCTTCA CGTGCTGGCC TTCTCTAAGA GCTGCCACTT	970	SCCTGGGCC AGTGGCCTGG AGACCTTGGA CAGCCTGGGG GGTGTCCTGG
092	CCAAACAGA	810	CTGACCTTA	098	CACCAGTAT	910	ACCTCCGGG	096	SCCTGGGCC

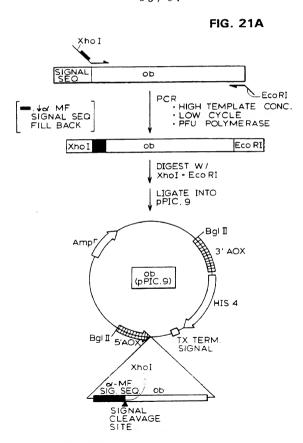
FIG. 20E

1050	GCTGCAGGG	1100	GGTGCTGAGG STOP	1150	AGGAACTCTG	1200	CACCCTTAT		1249	TTCCAAAGG
1040	CCCTGAGCAG	1090	CTCAGCCCTG	1140	CGTTAAGGGA	1190	ATTGCATGGA		1240	TAAGCCACTC
1030	AAGUTTCAGG CTACTCCACA GAGGTGGTGG CCTGAGCAG GCTGCAGGG	1080	TCTCTGCAGG ACATGCTGTG GCAGCTGGAC CTCAGCCCTG GGTGCTGAGG STOP	1130	CCTIGAAGGT CACTCTTCCT GCAAGGACTA CGTTAAGGGA AGGAACTCTG	1180	GCTITCCAGG TATCTCCAGG ATTGAAGAGC ATTGCATGGA CACCCTTAT		1230	CCAGGACTOT GTCAATTTCC CTGACTCCTC TAAGCCACTC TTCCAAAGG
1020	CTACTCCACA	1070	ACATGCTGTG	1120	CACTCTTCCT	1170	TATCTCCAGG	HOB 2G R	1220	GTCAATTTCC
1010	AAGCTTCAGG	1060	TCTCTGCAGG	1110	CCTTGAAGGT	1160	GCTTTCCAGG		1210	CCAGGACTCT

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MOU	SE OB ST	ומורדדום	=			
MOU	SE UB SI	RUCTUR				
!st ex	1st intr	2nd ex	2nd intr	3rd exon		
	_////////////					_
		start			stop	
						FIG. 201
HUMA	an ob st	RUCTUR	E			
	1:	st exon	1st intr	2nd exon		
	A	TG			TGA	
		tart			stop	

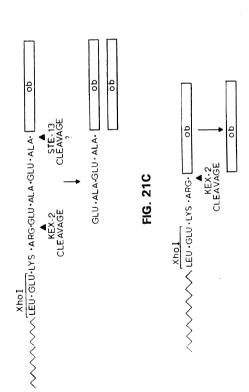
FIG. 20G



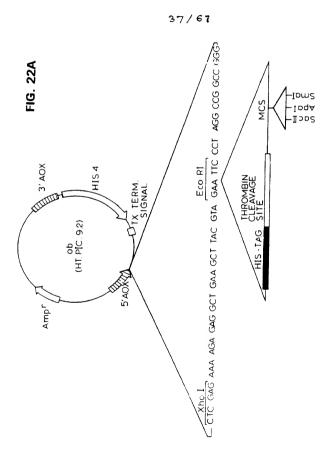
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FIG. 21B

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(FOLLOWING THROMBIN CLEAVAGE) 90 qo GLY · SER · PRO · A -MF SIG SEO GLU- ALA HIS . TAG THROMBIN CLE AVAGE STE-13 CLEAVAGE KEX-2 CLEAVAGE

FIG. 22B

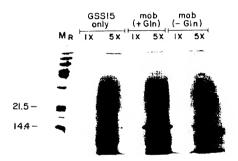


FIG. 23A

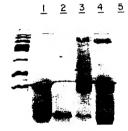


FIG. 23B

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PCT/US95/10479

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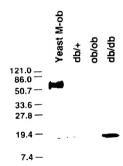


FIG. 24A



FIG. 24B

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recombinant ob (ng) wt 0.01 0.1 0.5 2.0 15.0

19.4 —

FIG. 24C



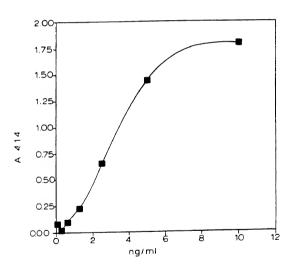
FIG. 24D



FIG. 25A

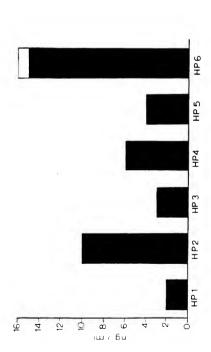
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FIG. 25B



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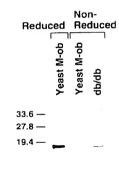
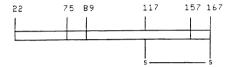


FIG. 26A

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Human ob



Peptide	Mass(Da)				
	Expected	Observed			
22-167	16,024	16,024 ± 3			
22-75	5936.9	5936.6 ± 1			
76-89	1562.7	N.D.			
90-167	8434.5	8435.6 ± 1			
158-167	1131.9	N.D.			

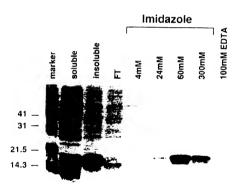


FIG. 27

0b/0b

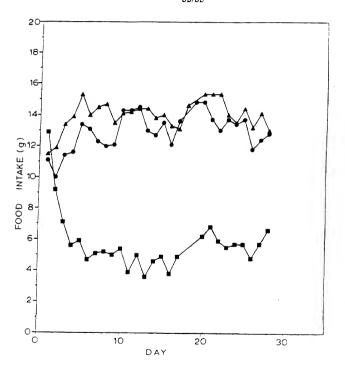


FIG. 28A

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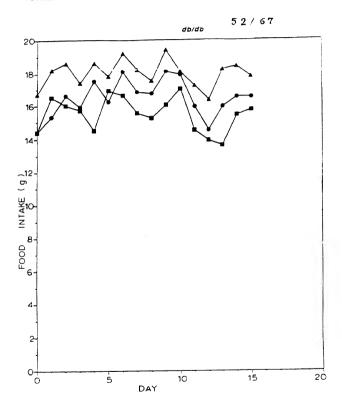


FIG. 28B

SUBSTITUTE SHEET (RULE 26)

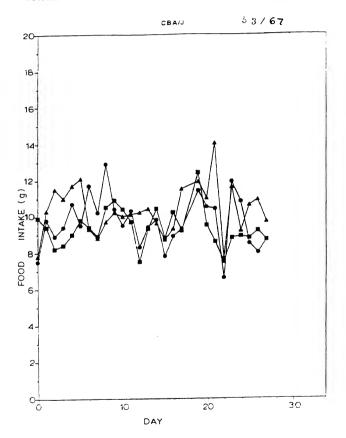


FIG. 28C

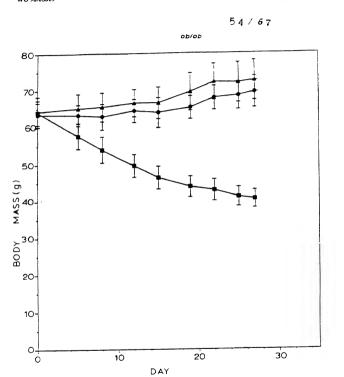


FIG. 28D



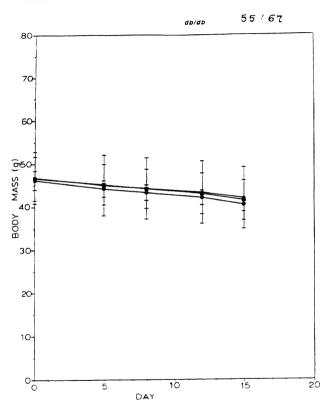


FIG. 28E

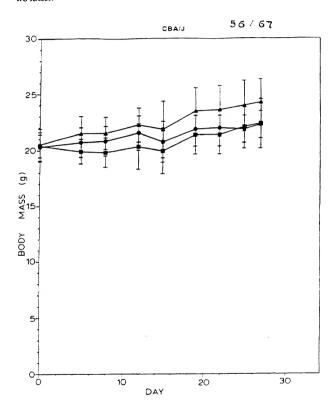
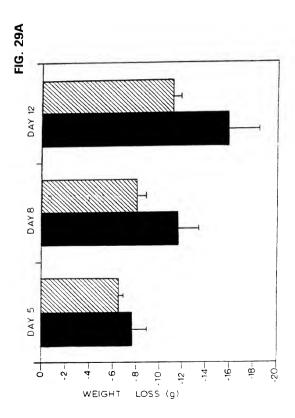


FIG. 28F



SUBSTITUTE SHEET (RULE 26)





FIG. 29B

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1G. 29C

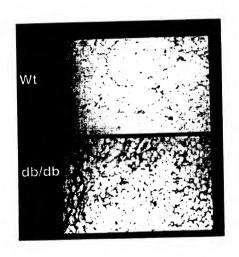


FIG. 30

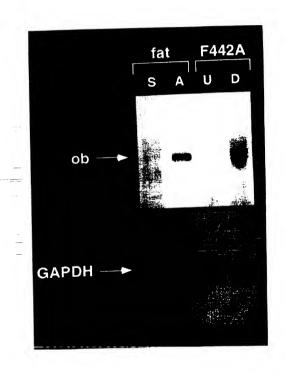


FIG. 31

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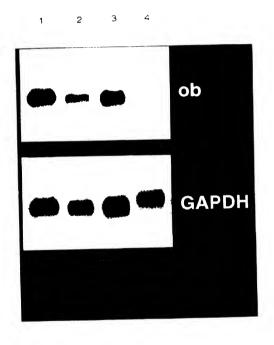


FIG. 32A

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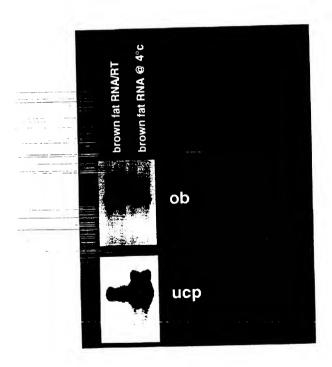


FIG. 32B

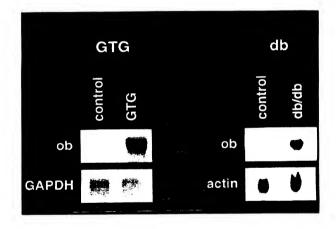


FIG. 33

